THE ELIMINATION OF UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS *IN VITRO*.

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DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisors. No part of this thesis has been, or will be, submitted for any other degree, diploma or qualification.

Zoë Anne Hewitt
December 2005

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ABSTRACT

Stem cells have been a focus of growing scientific interest both as a tool for studying early development and as a potential source of transplantable cells for regenerative medicine. Since their derivation in 1998, human embryonic stem (ES) cells have received much of this attention principally because of their ability to proliferate seemingly indefinitely *in vitro* and to differentiate along multiple lineages, essentially giving rise to every cell in the body (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998). However, before human ES-derived transplantation becomes a reality for patients, problems associated with prolonged and functional engraftment, histocompatability, homogeneous cell populations, and the risk of tumours arising from the accidental transplantation of undifferentiated human ES cells, must first be addressed.

Despite the risk of malignancy associated with undifferentiated human ES cells, their indefinite growth in culture has provided an opportunity to manipulate their fate. This thesis has investigated the possibility of using the exogenous cell surface markers; the Galα1-3Galβ1-4GlcNAc-R (α-gal) epitope and the murine major histocompatibility complex (MHC) class I molecule H2-K^k, under the transcription control of the human telomerase reverse transcriptase (hTERT) promoter to selectively identify undifferentiated human ES cells for selective elimination. Provided within is evidence to show the successful genetic manipulation of

undifferentiated human ES cells with α1,3galactosyltransferase (α1,3Gal) gene, providing comparable cell surface expression of the α-gal epitope with endogenous α-gal expression on the ovine foetal fibroblast cell line (PDFF). However, the use of the H2-K^k gene as a potential lysis epitope was not so successful. Despite successful integration and transcription of the H2-K^k gene, cell surface expression of the H2-K^k epitope was not achieved, and proof of protein translation could not be found.

Expression of the α-gal epitope on undifferentiated human ES cells elicits a severe, yet highly specific cytolytic response; on average 95% of the transgenic human ES cells were lysed compared with just 8-12% of wild type non-expressing H9 cells, when exposed to human serum containing active-complement. In addition, when transgenic human ES cells were differentiated the α-gal epitope was down regulated, in the same manner as established markers of undifferentiated human ES cells (TRA-1-81 and SSEA-4). Following differentiation the transgenic α-gal expressing cell line (M2) survived exposure to active serum-complement.

This novel system for selective ablation could potentially provide natural immune protection, through the presence of circulating antibodies to α-gal that would protect graft-recipients against the presence of, or de-differentiation of, human ES-cell derivatives following engraftment.

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LIST OF ABBREVIATIONS

AO	Acridine Orange
APC	Antigen Presenting Cells
β-2M	β-2 Microglobulin
bp	Base Pair
BSA	Bovine Serum Albumin
BS-IB$_4$	*Bandeiraea simplicifolia* isolectin B4 lectin (FITC-conjugated) specific for gal epitope
cDNA	Complementary DNA
CM	Conditioned Media
DAF	Decay Accelerating Factor (CD55)
Dapi	4',6 diamidino-2-phenylindole
dH_2O	Deionised Water
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbeccos Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP's	Deoxyribonucleoside triphosphates
dpc	Days Post Coitum
DTT	Dithiothreitol
EB	Embryoid Body
EC	Embryonal Carcinoma
EG	Embryonic Germ
ES	Embryonic Stem
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorting

FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FSC	Forward-Angle Light Scatter
g	Acceleration due to gravity
GCV	Ganciclovir
gDNA	Genomic DNA
GFP	Green fluorescent protein
GMEM	Glasgow's Minimum Essential Medium
hBFGF	Human Basic Fibroblast Growth Factor
HEK 293	Human Embryonic Kidney Cell Line 293
HLA	Human Leukocyte Antigen
HSC	Haematopoietic Stem Cells
HSV-tk	Herpes Simplex Virus – thymidine kinase
IFN-γ	Interferon-Gamma
IPCC's	Insulin Producing Cell Cluster
KO-DMEM	Knockout Dulbeccos Modified Eagle's Medium
KO-SR	Knockout Serum Replacement
LB Medium	Luria-Bertani bacterial medium
LIF	Leukaemia Inhibitory Factor
m	Milli
M	Molar
ml	Millilitre
MACS	Magnetic Activated Cell Sorting
MAPC	Multipotent adult progenitor cell
MCP	Membrane Co-Factor Protein (CD46)
MEF	Mouse Embryonic Fibroblasts
MHC	Major Histocompatibility Complex
MIRL	Membrane Inhibitor of Reactive Lysis (CD59)
mRNA	Messenger RNA
neo	Neomycin phosphotransferase
NHS	National Health Service
NK	Natural Killer Cells
NOD	Non-Obese Diabetic Mice

OD	Optical density
OPC	Oligodendrocyte Progenitor Cells
P	Passage
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDFF	Primary Dorset Foetal Fibroblasts
PEV	Position Effect Variegation
PGC	Primordial Germ Cell
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
RA	Retinoic Acid
RIA	Radio-Immuno Assay
RIGS	Repeat Induced Gene Silencing
rpm	Revolutions per minute
R-PE	R-phycoerythrin
RPMI 1640	Roswell Park Memorial Institute Medium 1640
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SCID	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
SR	Spontaneous Release
SSC	Side-Angle Light Scatter
SSEA	Stage specific embryonic antigen
TE	Tris/EDTA
TED	Trypsin EDTA Solution
TEG	Trypsin EGTA Solution
TR	Total Release
µl	Micro-litres
µm	Micro-metres
µM	Micro-molar
UV	Ultra-Violet
U	Units

CHAPTER 1 INTRODUCTION

1.1 The Promise of Regenerative Medicine

Stem cells have been a focus of growing scientific interest both as a tool for studying the earliest events of development and as a potential source of transplantable cells to regenerate missing or damaged tissues, or to restore functions lacking in individuals with acquired or inherited disorders. The isolation of embryonic stem (ES) cells, which display a unique ability to differentiate into all cells of the adult body, has heightened this interest over the last two decades (Reubinoff *et al,* 2000; Thomson *et al,* 1998; Evans & Kaufman, 1981; Martin, 1981).

1.1.1 The Differentiation Potential of ES cells into Therapeutically Relevant Lineages.

Within the last decade, a number of important advances towards obtaining therapeutically useful cell types have been achieved through studies of directed *in vitro* differentiation. The majority of this work has focused on the differentiation of murine ES cells as a model for therapeutically useful cell types to treat a number of the world's most debilitating diseases, a selection of which can be found in Table 1.1.1. Initially, differentiation was achieved using spontaneous differentiation protocols and genetic manipulation to isolate and enrich for specific sub-sets of cells (Miyashita *et al.,* 2002; Perlingeiro *et al.,* 2001; Klug *et al.,* 1996). However, strategies have since been developed, based on an understanding of embryo development *in vivo*, which utilise the addition of specific growth factors to encourage homogenous differentiation into specific cell types (Yusas *et al.,* 2005; Kawaguchi *et al.,* 2005; Kim *et al.,* 2002).

The derivation of human ES cells in 1998 has had exciting implication for the field of regenerative medicine (Reubinoff *et al,* 2000; Thomson *et al,* 1998). Extrapolation of the advances made with murine ES cells have indicated that human ES cells will differentiate, in most respects, in a similar manner, although there may be slight differences in the exogenous cues. Table 1.1.2 provides a selection of significant advances made with human ES cells paralleling those made with murine ES cells (Table 1.1.1).

Table 1.1.1: A summary of some of the most recent advances in the development of therapeutically useful cells from murine ES (mES) cells, providing a brief description of the works and references.

Disease	Cell type	Evidence	Reference
Spinal Cord Injury	Neural stem cells and neurones	ES-derived neurones were shown to stimulate recovery of the spinal cord following transplantation into rats suffering from weight drop injury.	McDonald *et al.*, (1999)
Parkinson's Disease	Dopaminergic Neurones	Dopaminergic neurones, differentiated through EBs, released significant levels of dopamine and sustained trains of action potentials characteristic of mature neurones.	Lee *et al.*, (2000),
		Expression of Nurr1 prior to differentiation increased the occurrence of dopaminergic neurones (78%). Transplantation resulted in a significant ($P<0.001$) improvement in rotational behaviour compared to that observed from wild type ES cell-derived dopaminergic neurone transplants.	Kim *et al.*, (2002) &
		ES cells differentiated into dopamine-releasing neurones *in vivo*, showed recovery from rotational behaviour over time.	Björklund *et al.*, (2002)
Multiple Sclerosis (MS)	Oligo-dendrocytes	Functional engraftment of oligodendrocytes, derived from mES, in md rats. Six out of 9 rats showed donor derived myelin sheaths and showed evidence of cell migration away from the site of injection.	Brüstle *et al.*, (1999)
Loss of sight	Retinal progenitors	Directed differentiation of retinal progenitor neurones through co-culture with PN1 retinal cells led to 6% of the progenitors expressing rhodopsin, a specific photoreceptor regulatory gene.	Zhao *et al.*, (2002)
Hearing Loss	Sensory receptors of the inner ear (Hair cells)	ES cells differentiated into inner ear progenitors, when transplanted into chicken embryos resulted in integration into the developing cochlear hair cell layer, preferentially at sites of slight injury to the epithelium layer. Donor cells expressed specific markers of hair cells and hair bundles in 30/50 cases.	Li *et al.*, (2003)
Diabetes	Insulin producing cells (β-cells)	ES derived insulin-producing cells clusters (IPCCs), maintained similar topology and organisation to normal pancreatic islets when injected into diabetic mice. Insulin production was lower than primary pancreatic islets, but this report has improved on the early attempts.	Lumelsky *et al.*, (2001) &
		Transplantation of IPCCs into a diabetic mouse model showed maintenance of body weight and extended survival. When grafts were removed, the mice entered a rapid state of hyperglycemia and died.	Hori *et al.*, (2002)
Heart Disease	Cardio-myocytes	Spontaneously differentiated mES cells were enriched for cardiomyocytes by the presence of a selection cassette. When engrafted into the hearts of adult dystrophic mice, they formed stable intracardiac grafts, for up to 7-weeks, in mice.	Klug *et al.*, (1996) &
		Directed differentiation through the inhibition of BMP signalling with Noggin treatment increased production of cardiomyocytes 100-fold, 95.3% of EBs were observed to beat after 10 days.	Yuasa *et al.*, (2005)

Cont.

Blood Diseases	Erythroid cells, myeloid cells including B and T Lymphocytes	Hematopoietic progenitors isolated from EBs gave rise only to primitive erythroid cell types *in vitro*. When clonally injected into irradiated mice, they gave rise to multiple myeloid cell types as well as B- & T-lymphocytes, which under went maturation *in vivo*. A sub-population of flk1+/CD45- pre-hematopoietic stem cells was identified following the co-culture of mES with OP9 stromal cells. Specific differentiation into B- and T-lymphocytes was initially poor but was elevated following re-aggregated thymic organ cultures.	Perlingeiro *et al.*, (2001) & de-Pooter *et al.*, (2003)
Infertility	Germ cells (Sperm and Oocytes)	Male and female mES cells were shown to spontaneously differentiate into oogonia after 50 days in 2D culture. These oogonia entered meiosis and recruited adjacent cells to form follicle-like structures and later developed into blastocysts. *In vitro* differentiation (EBs) of ES cells into PGC,s followed by co-culture with dissociated male gonads resulted in the formation of testicular tubes and mature sperm in the lumens, when transplanted back into host testis capules. Controls containing only gonadal aggregates (no ES-derived PGCs) resulted in tubules but no sperm. ES-derived sperm were used to fertilise oocytes through intracytoplamic injection: 50% went to the 2-cell stage, of which 20% formed blastocytes.	Hűbner *et al.*, (2003), Toyooka *et al.*, (2003) & Geijsen *et al.*, (2003)
Liver Disease	Hepatocytes	Spontaneous differentiation of hepatocytes was achieved *in vitro* (through EBs) and *in vivo* (through teratomas formation). Hepatocytes isolated from teratomas and expanded *in vitro* were found to have characteristic hepatocyte metabolism and when transplanted back into the spleens of carbon tetrachloride treated mice they migrated to the liver, were indistinguishable from host hepatocytes and functioned to reduce ammonia levels towards normal.	Miyashita *et al.*, (2002) & Yamamoto *et al.*, (2003)
Bone Disease	Osteoblasts	Differentiation of osteoblasts from EBs, in the presence of known osteogenic factors resulted in expression of osteogenic markers, calcium deposition and classic bone morphology. Treatment of EBs with retinoic acid, before addition of growth factors resulted in specific bulk differentiation of osteoblasts, chondrocytes and adipocytes from a common progenitor.	Buttery *et al.*, (2001) & Kawaguchi *et al.*, (2005)

Table 1.1.2: A summary of some of the most recent advances in differentiation of therapeutically useful cell types from human ES (hES) cells, providing a brief description of the works and references.

Disease	Cell Type	Evidence	Reference
Neuro-degenerative diseases	Neurones, astrocytes & oligo-dendrocytes	Retinoic acid (RA) and β-nerve growth factor significantly enhance the differentiation of hES cells into neurones *in vitro*. RA led to maturation of neurones that expressed dopamine and serotonin receptors.	Schuldiner *et al.*, (2001),
		Neural-spheres differentiated from hES cells, were directed to differentiate into all 3 neural cell lineages by the addition of extrinsic growth factors. When hES-derived neural progenitors were injected into mice, they differentiated appropriately and migrate along established developmental tracks	Reubinoff *et al.*, (2001) & Zhang *et al.*, (2001)
		Undifferentiated hES cells modified to express GTPCH I, a gene involved in tyrosine hydroxylase activity (dopaminergic neurones) underwent *in vivo* differentiation when transplanted into rat models of Parkinson's disease. Cells survived for up to 6 weeks and improved rotational behaviour without the formation of tumours.	Park *et al.*, (2003)
		In vitro derivation of oligodendrocyte progenitors (OPC's) from hES cells, which enhance remyelination and promoted motor function in rats following spinal cord injury, without the formation of teratomas after 1 year *in situ*.	Keirstead *et al.*, (2005)
Diabetes	Insulin producing cell clusters (IPCC)	Modifying the protocol described by Lumelsky *et al.*, (2001) for mES cells, by reducing the level of glucose in the medium and allowing the cells to aggregate prior to differentiation, hES cells were differentiated into IPCCs that secreted a 30-fold increase in insulin, and that continued to proliferate in their differentiated state for up to a month. However, the gene expression profile of hES-derived IPCC's indicated that they were immature compared to those produced by monolayer cultures.	Segev *et al.*, (2004)
Blood Diseases	Haematopoietic cells	Undifferentiated hES cells were co-cultured with irradiated mouse bone marrow cells (S17 cells) and allowed to spontaneously differentiate. From the mixed population CD34+ cells were isolated and plated in methylcellulose culture medium where they differentiated into colony forming units representing macrophages, granulocytes, megakaryocytes and erythrocytes.	Kaufman *et al.*, (2001)

Cont.

Heart Disease	Cardio-myocytes	Directed differentiation of hES cells through co-culture with visceral-endoderm cell lines resulted in cardiomyocytes that had classical morphology, expressed characteristic markers and displayed action potentials that were comparable to those observed in 16-week-old foetal atrium and ventricle tissue. Cardiomyocytes, differentiated *in vitro* through EBs and the addition of 5-aza2'-deoxycytidine, responded appropriately *in vitro* to various cardioactive drugs. The use of Percoll gradient separation resulted in a 4-fold enrichment of differentiated cardiomyocytes, which also contained a subpopulation that appeared to be proliferative following reintroduction into culture.	Mummery *et al.*, (2002) Xu *et al.*, (2002)
Liver Disease	Hepatocytes	Differentiation of hepatocyte-like cells from hES cells through treatment with sodium butyrate. Isolation of hepatocyte-like cells was low (10-15% of the total culture) and cell death was high, however, those cells, which survived, had morphology similar to primary hepatocytes and 70-80% of them expressed liver-associated proteins.	Rambhatla *et al.*, (2003) & Lavon *et al.*, (2004)
Bone Disease	Osteoblasts	Differentiation of committed osteoblasts from EBs in the presence of known osteogenic factors resulted in expression of osteogenic markers, calcium deposition and classic bone morphology. *In vivo* differentiation of mineralised tissue from hES cells transplanted into mice in poly-$_{D}$, $_{L}$-lactide scaffold, without tumour formation.	Sottile *et al.*, (2003) Bielby *et al.*, (2004)

1.2 The Limitations of Regenerative Medicine

While the promise of human ES-derived regenerative medicine has exciting implications for modern medicine, there remain a number of significant challenges before cell-transplantation can become a reality for patients. The challenge of differentiating ES cells so that they are available in sufficient number to achieve adequate engraftment to improve physiological conditions over long periods of time has already received significant attention (as discussed 1.1.1). However, while a number of reports have now demonstrated the ability of ES cells to differentiate and incorporate into host tissues (Keirstead *et al.,* 2005; Reubinoff *et al.,* 2001; Zhang *et al.,* 2001; McDonald *et al.,* 1999; Klug *et al.,* 1996) the evidence for this engraftment has been based upon the expression of characteristic markers, 4-8 weeks after transplantation. Recently, there has been the first published study that has assessed the ability of stem cell-derived cell grafts to provide long-term functional improvement (Keirstead *et al.,* 2005).

In this report, Keirstead *et al.,* (2005) demonstrate derivation of functional oligodendrocyte progenitors cells (OPC's) from human ES cells. When transplanted into rat spinal cords, 7 days post injury, these cells not only survived, migrated, matured and incorporated into host tissue, as indicated by morphology and the expression of characteristic markers, they also enhanced remyelination of axons and promoted motor function, as soon as 1-week post transplant (Keirstead *et al.,* 2005). Remyelination was assessed over 12-months and indicated that OPC-transplanted rats displayed approximately twice the amount of remyelination one year after transplantation compared to sham transplanted control rats. Interestingly, the authors

described the existence of a therapeutic window, restricted to the early post-injury period, in which this therapy was effective. OPC's transplanted 10-months after spinal cord injury showed cell survival and migration but there was no evidence of remyelination or improved locomotive ability after a year *in situ* (Keirstead *et al.,* 2005).

One of the main restrictions in determining functional improvement and long-term survival of ES cell grafts is that the majority of the models of human diseases are rodent models, with short life spans. However, if ES cells could be derived from farm animals, showing the same success in differentiation, it would significantly enhance the study of cell transplantation. Attempts have been made to this end, and ES-like cells have been derived from cows and pigs (Wang *et al.,* 2005; Picard *et al.,* 1990; Li *et al.,* 2004). However, as yet pluripotent cells with indefinite proliferative capability have not been isolated. The increased life span of farm animals would allow for the analysis of homologous transplants, of specific cells types, over several years, which would help to determine the benefits, and any possible long-term problems, associated with transplantation of ES-derived cells.

Currently the major obstacle associated with organ and tissue transplantation, is the issue of histocompatiblity, which must be addressed before ES-derived cell grafts can be used therapeutically. Therapeutic cloning, the derivation of patient-specific embryos, is one suggested method by which graft rejection of transplanted human ES cell derivatives might be prevented. Initially it was considered that this prevention strategy would be difficult to achieve since the frequency of successful cloning

(success being determined in terms of live births) has been shown to be very low, ranging from 3.4% in sheep to as low as 0.3% in Pigs (Wilmut *et al.,* 1997; Polejaeva *et al.,* 2000, respectively). However, researchers have recently reported the derivation of human ES cells from embryos produced by somatic nuclear transfer (NT) (Hwang *et al.,* 2004) and have indicated a significantly better than expected success rate, increasing from 3.3% success in the first report (Hwang *et al.,* 2004) to over 35% a year later (Hwang *et al.,* 2005). The group indicated that the success of NT-human ES cell derivation was reduced when using oocytes donated by women over the age of 30, which could have significantly limited this technology from becoming a widely practiced clinical application. However, there is evidence to suggest that the number of "banked" ES cell lines that would be required to match the human leukocyte antigen (HLA) for the majority of human genotypes, with the exception of individuals with rare genotypes, could be achieved with only 150 blood matched donors, or as few as 10 highly selected donors for the most common HLA haplotypes (Taylor *et al.,* 2005). The authors went on to show that banking greater than 150 blood matched donors would not significantly increase the ability to match patient HLA. Together with the report of Hwang *et al.,* (2005) these data suggest that somatic nuclear transfer could be a plausible technique to overcome the issue of histocompatibility, although studies into the proliferation, differentiation and stability of these cells need to be performed.

A further cause for concern with ES-derived cell transplants is the potential for immune pathology caused by activation of lymphocytes or hyperfunction, where cells trying to supply a missing function at a regulated level, such as insulin, fail and

over produce. Selective ablation strategies have been developed (see section 1.5) which could be used to target the elimination of a graft in the event of post-transplant complications (Schuldiner *et al.,* 2003; Fareed & Moolten, 2002).

In addition to the risk of immune pathology and hyperfunction, there is also a serious risk that transplanted cells may result in the formation of malignancies either as a result of tissues maintaining growth potential or arising from the unintentional transplantation of undifferentiated human ES cells within a therapeutic population. These potentially malignant cells may develop, or persist, during the production of a transplantable cell line, or could arise during the stage of rapid cell proliferation, which will be required to obtain adequate numbers of cells for transplantation (Fareed & Moolten, 2002). Murine models have shown the potential of undifferentiated human ES cells to differentiate inappropriately after transplantation, forming teratomas or even highly malignant teratocarcinomas (Reubinoff *et al,* 2000; Thomson *et al,* 1998) and therefore, it is essential to address this risk before ES derived-cells enter the clinic.

This thesis describes an attempt to address concerns of tumorigenecity from human ES-derived regenerative medicine, before this therapy enters the clinic. Presented within is an investigation into strategies which selectively eliminate undifferentiated human ES cells, utilising restricted expression (see section 1.3.5.2) of a variety of different cell surface markers (see section 1.6) to undifferentiated human ES cells and showing the application of an appropriate cell selection or ablation technique (see section 1.7).

1.3 Introduction to Stem Cells

Stem cells have been defined by their ability to divide asymmetrically: one daughter cell remains as a stem cell, to maintain the original stem cell population, while the other is directed down a specific developmental pathway that commits it to mature into a single, or one of many, specialised cell types.

Conventional wisdom holds that adult (or somatic) stem cells are constrained in their developmental potential to their tissue of origin and hence were thought to be restricted in their therapeutic potential. Conversely, embryo derived pluripotent stem cells can give rise to essentially any cell type in the animal body (Donovan & Gearhart, 2001; Thomson & Odorico, 2000; Reubinoff *et al*, 2000; Keller & Snodgrass, 1999; Thomson *et al*, 1998 Thomson et al, 1995; Evans & Kaufman, 1981; Martin, 1981). At present, three types of embryo-derived multipotent stem cell lines have been isolated from mammals – embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas (Kleinsmith & Pierce, 1964); embryonic germ (EG) cells derived from the primordial germ cells (PCG's) of post-implantation embryos (Shamblott *et al*, 1998; Matsui *et al*, 1992); and embryonic stem (ES) cells derived from pre-implantation embryos (Reubinoff *et al*, 2000; Thomson *et al*, 1998; Thomson et al, 1995; Evans & Kaufman, 1981; Martin, 1981).

1.3.1 Multipotent Adult Stem Cells

Recently the notion that adult stem cells are constrained in their lineage potential to the tissue of their origin has been challenged. It has been reported that adult stem cells have an inherent plasticity that allows them to respond to extrinsic signals,

raising hopes that patient-derived adult stem cells may be used to treat a wide variety of diseases (reviewed by Bianco *et al.,* 2001; Brazelton *et al.,* 2000; Ferrari *et al.,* 1998). However, although the plasticity of adult stem cells has been reported by many groups and for many different tissues, reports of stem cell plasticity have been criticised. Most of the objections to stem cell plasticity seem to revolve around the low frequency at which it occurs and also that the majority of the reported data has been based on pools of bone marrow derived stem cells which may contain several tissue specific progenitor cells which co-exist (reviewed by Wagers & Weissman 2004 and Lakshmipathy & Verfaillie 2004).

Wagers *et al.,* (2002) have evaluated the *in vivo* cell fate of haematopoietic stem cells (HSC) through the generation of chimeric mice produced by transplanting a single green fluorescent protein (GFP) tagged HSC into lethally irradiated recipients (Wagers *et al.,* 2002). The authors reported that out of over 15,000,000 cells examined only 8 cells (1 in the brain and 7 in the liver) were GFP positive and not of the haematopoietic lineage (Wagers *et al.,* 2002). In agreement with other reports (Terada *et al.,* 2002; Ying *et al.,* 2002) Wagers *et al* (2002) proposed that HSC plasticity could be explained by a spontaneous fusion event between the stem cell and a non-haematopoietic cell. Spontaneous fusion, as with stem cell plasticity does not occur at a high frequency but these observations have been seen repeatedly in different groups (Terada *et al.,* 2002; Ying *et al.,* 2002), including our own (Pells *et al.,* 2002).

In recent years there have been reports of adult stem cells with extensive potential for differentiation, into multiple lineages; multipotent adult progenitor cells - MAPC's (Jiang *et al.,* 2002) and marrow isolated adult multilineage inducible (MIAMI) cells (D'lppolito *et al.,* 2004). Both reports indicated the isolation of a stem cell population from the bone marrow which had the ability to grow for extended periods of time, expressing a number of markers found to be expressed by ES cells (Oct-4, Rex-1 and telomerase). Furthermore, these cells could differentiate *in vitro* into multiple cell types representing all three germ layers. In addition, MAPC's have been shown, at the single cell level, to contribute to most, if not all, somatic cell types when injected into an early blastocyst (Jiang *et al.,* 2002). The potential of multipotent adult stem cells (MAPC's and MIAMI cells) is very exciting, however, until independent laboratories have repeated these results, their true potential as a source of cells for therapy remains unclear.

1.3.2 Embryonal Carcinoma Cells

The potential of multipotent stem cells was first realised in the early 1960's with the isolation of embryonal carcinoma (EC) cells (Martin & Evans, 1974; Evans, 1972; Kleinsmith & Pierce, 1964). EC cells are responsible for the formation of highly malignant germ cell tumours, known as teratocarcinomas, which are characterised by the presence of disorganised tissues representative of all three germ layers: ectoderm, endoderm and mesoderm, and a single population of multipotent stem cells from which they were derived (Kleinsmith & Pierce, 1964). Characterisation of EC cells has shown that they have an indefinite replicative capacity (self-renewal) and that transplantation of a single EC cell resulted in the formation of a new teratocarcinoma

(Kleinsmith & Pierce, 1964). There is also evidence to suggest that certain established EC cell lines can undergo normal embryonic development when injected back into a mouse blastocyst, giving rise to chimeras with tissues derived from both the host blastocyst and the EC cells, suggesting that EC may be pluripotent (Waters & Rossant 1986; Illmensee & Mintz 1976; Papaioannou *et al.,* 1975). However, such chimeras have been found to develop abnormally (Rossant & McBurney 1982; Papaioannou *et al.,* 1978), casting doubt on the potential of EC cells, and indicating that perhaps the risk associated with using tumour-derived, aneuploid cell lines (EC cells) would be inappropriate for therapeutic use in humans. Nevertheless, EC cells have played a fundamental role in furthering the field of regenerative medicine, since it was an understanding of the biology of EC cells and early embryo development that provided Martin Evans and Matt Kaufman and independently Gail Martin, with the necessary grounding to derive pluripotent stem cells (see section 1.3.4) directly from murine blastocysts (Evans & Kaufman, 1981: Martin, 1981).

1.3.3 Embryonic Germ Cells

A second source of embryo-derived multipotent stem cells was derived from the primordial germ cells (PGC) of post-implantation embryos. These stem cells, termed embryonic germ (EG) cells to reflect their origin, were derived at the point of PGC migration from the posterior primitive streak to the genital ridge. In the mouse this occurs at 8.5 days post coitum (dpc) (Matsui, *et al,* 1992) and in humans from embryos between 5-9 weeks post-fertilisation (Shamblott *et al,* 1998). As with EC cells, EG cells have been shown to have the capacity for self-renewal and have been shown to be multipotent (Shamblott *et al,* 1998; Matsui, *et al,* 1992). The potential

of murine EG cells has been shown both with the formation of teratocarcinomas and coat colour chimeras (Stewart *et al,* 1994; Labosky *et al,* 1994). However, there appears to be a window in which murine EG cells are pluripotent, which seems to be restricted to the early gestational period. For example, EG cells lines derived from PGC's of mouse strains 129/Sv and C57BL/6 at 8.5 dpc have been shown to successfully contribute to the germ-line of chimeras (Stewart *et al,* 1994; Labosky *et al,* 1994), while EG cells derived from the same C57BL/6 mouse strain but from PGC's at 12.5 dpc could not (Labosky *et al,* 1994). The reason(s) for this are not fully understood, although, it has been suggested that changes in methylation patterns and genetic imprinting, when compared to those of ES cells for example, may have an affect on the ability of some EG cells to contributed to the germ-line (Labosky *et al,* 1994). The potential of human EG cells has been demonstrated *in vitro,* differentiation of human EG cells gave rise to derivatives which expressed markers from all three embryonic germ layers, however, *in vivo* differentiation of human EG cells has not resulted in the formation of teratomas (Shamblott *et al,* 1998), possibly reflecting a later developmental status for EG cells then evidenced in ES cells for example (see section 1.3.4).

1.3.4 Embryonic Stem Cells

By comparing the properties of embryonic cells with those of established EC cell lines at varying stages of development, Evans and Kaufman found that the epiblast cells of the early post-implantation embryo (5.5dpc) most closely reflected the cell-surface antigen expression and protein synthesis patterns of EC cells. However, isolation and explantation of sufficient cells from embryos at the epiblast stage of

development was technically very challenging. To overcome this issue, pre-implantation blastocysts were induced into an experimental state of dormancy (diapause), by ovariectomy at 2.5dpc, allowing the embryo to hatch from the surrounding zona, while remaining free-floating in the uterine lumen. The suspension of implantation led to an increased cell number within the embryo without advancing embryo development and in turn made isolation of the epiblast more successful. These blastocysts were then cultured in conditions optimised for EC cells and provided the first karyotypically normal, truly pluripotent embryonic stem (ES) cell lines which appeared to be unrestricted in their proliferation (Martin 1981; Evans & Kaufman, 1981). The ability of murine ES cells to develop into specialised cell types, representative of all three embryonic germ layers, has been tested in three independent assays: *in vitro* differentiation, giving rise to cell derivatives expressing markers representative of all three germ layers (see Table 1.1.1); differentiation into teratomas or teratocarcinomas when placed in syngeneic adult or immunocompromised mice; and *in vivo* differentiation when introduced into the blastocoel cavity of a pre-implantation embryo (Donovan & Gearhart, 2001).

Following the successful isolation of ES cells from mouse (Evans & Kaufman, 1981; Martin, 1981), there are now a number of established primate cell lines from both Rhesus (Thomson et al, 1995) and Cynomolgus (Suemori *et al.,* 2001) monkey, marmoset (Thomson *et al.,* 1996) and human (Reubinoff *et al*, 2000; Thomson *et al*, 1998).

1.3.5 Human Embryonic Stem (ES) Cells

Human embryonic stem (ES) cells were first derived by James Thomson and his colleagues in 1998 (Thomson *et al.,* 1998). Embryos, donated by patients' consent, from IVF clinics, were cultured to the blastocyst stage and a total of 14 inner cell masses (ICM's) were isolated. From these, five independent Wisconsin human ES cell lines were established, three with a stable XY karyotype (H1, H13 and H14) and two with a stable XX karyotype (H7, and H9). Amongst the unique properties reported for ES cells, the ability to self renew is one of their defining characteristics, the other being the ability to differentiate into multiple cell types of the adult body. Thomson *et al.,* (1998) reported that all five of the Wisconsin cell lines grew in continuous culture for at least six-months, never experiencing replicative crisis; that they differentiated (*in vitro* and *in vivo*) into multiple cell types from all three germ layers; maintained their normal karyotype and expressed high levels of telomerase activity (Thomson *et al.,* 1998), indicating that these lines were in fact ES cells.

1.3.5.1 *In Vitro* Culture Techniques

The early-derived human ES cell lines were maintained in culture conditions, which paralleled those defined for murine ES cells, on a feeder-layer of mouse embryonic fibroblasts (MEF), in medium that was supplemented with serum and leukaemia inhibitory factor (LIF) (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998). However, unlike murine ES cells, human and non-human primate ES cells were found to be unresponsive to exogenous LIF, when removed from their feeder-cells, resulting in spontaneous differentiation (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998; Thomson *et al.,* 1995).

Initially, human ES cells, like non-human primate ES cells were passaged using a combination of mechanical and collagenase IV or dispase disaggregation, which maintained cells in small clumps at passage (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998; Thomson *et al.,* 1996; Thomson *et al.,* 1995). Human ES cells were initially maintained in small clumps at passage based on experience with non-human primate ES cells, which had indicated significantly improved cell survival compared to passage as a single cell suspension (Thomson *et al.,* 1995). However, while attempting to prove the pluripotency of clonal human ES cell, Amit *et al.,* (2000) found that cell survival of single cell suspensions of human ES cells was significantly improved by serum-free culture, supplemented with recombinant human basic fibroblast growth factor (hbFGF). They also demonstrated that serum-free medium that lacked hbFGF resulted in spontaneous differentiation of human ES cells, yet addition of hbFGF alone, or together with LIF, was insufficient to maintain human ES cells in an undifferentiated state, in the absence of feeder-cells (Amit *et al.,* 2000). These data suggested a number of things; 1) that a component of bovine serum was potentially toxic to undifferentiated human ES cells when they were exposed as single cells, 2) serum-free culture significantly increased survival, but reduced proliferation, without the addition of hbFGF, 3) hbFGF was not sufficient to maintain ES cells in an undifferentiated state and 4) that the feeder layer was essential for the maintenance of self-renewal (Amit *et al.,* 2000).

By 2001, a new method of human ES cell culture had been described (Xu et al., 2001), which substituted culture of ES cells on feeder-cell layers, with a synthetic

extra-cellular matrix (Matrigel) using serum-free medium pre-conditioned by MEFs in the presence of hbFGF. It was this basic method of culture that was used in the present study (see section 2.4.1). However, the ultimate goal for the field of regenerative medicine is to use derivatives of ES cells to treat degenerative disease. There are concerns that since current human ES cells lines have been derived on or cultured using either mouse feeders or MEF conditioned medium, mouse laminin (main component of matrigel) or serum, there is the possibility of cross species transfer of infectious agents. Therefore, the regulatory bodies have perceived current human ES cell lines to be xenografts. Consequently, extensive research has been undertaken to overcome this issue by defining derivation and culture conditions which do not involve contact with animal cells or animal products (Amit *et al.,* 2004; Amit *et al.,* 2003; Cheng *et al.,* 2003; Hovatta *et al.,* 2003; Richards *et al.,* 2003; Richards *et al.,* 2002). Recently, the first human ES cell lines have been successfully derived in completely defined conditions (in the absence of serum and serum replacement), and free from all animal products. Importantly these ES cell lines have been shown to be comparable to human ES cells derived using standard conditions, in terms of their marker expression, chromosomal stability and differentiation potential (Fletcher *et al.,* in preparation; Li *et al.,* 2005).

1.3.5.2 Characterisation of Human ES cells

Since the derivation of murine ES cells, much has been learnt of their gene expression profile, which has been shown to be closely related to the gene expression profile of the inner cell mass (Pelton *et al.,* 2002). Those characteristic markers

include telomerase activity, expression of the Oct3/4, Nanog, Sox-2 and Rex-1 genes and cell surface expression of the carbohydrate epitopes SSEA-1 and -3 and the liver/bone/kidney isozyme of alkaline phosphatase (reviewed by Ginis *et al.,* 2004). It is interesting to note that the characteristics of murine ES cells are similar to those observed for human EC and ES cells, however, there are also a number of differences, such as the expression of SSEA-1 and lack of TRA-1-60 and TRA-1-81 staining on murine ES cells. This section will compare the characteristic markers of murine ES cell with those of human EC and human ES cells.

Telomerase activity and self-renewal

DNA replication requires DNA polymerase, which functions by extending an RNA primer in a 5' to 3' direction (Figure 1.3.1). After initiation of DNA synthesis the RNA primers are removed and DNA polymerase extends the newly synthesised DNA across the gaps left by the primers, with the help of DNA ligase, to complete strand synthesis. However, when the RNA primer is removed from the far 5' end of the chromosome, DNA polymerase cannot complete the strand, as it has nothing to prime from, resulting in a shorter replicate strand (Figure 1.3.1). Telomere shortening, as it is known, occurs at each round of replication and has thus been proposed to act as a mitotic clock, signalling the progression of a cell towards the end of its lifespan (Allsopp *et al.,* 1992).

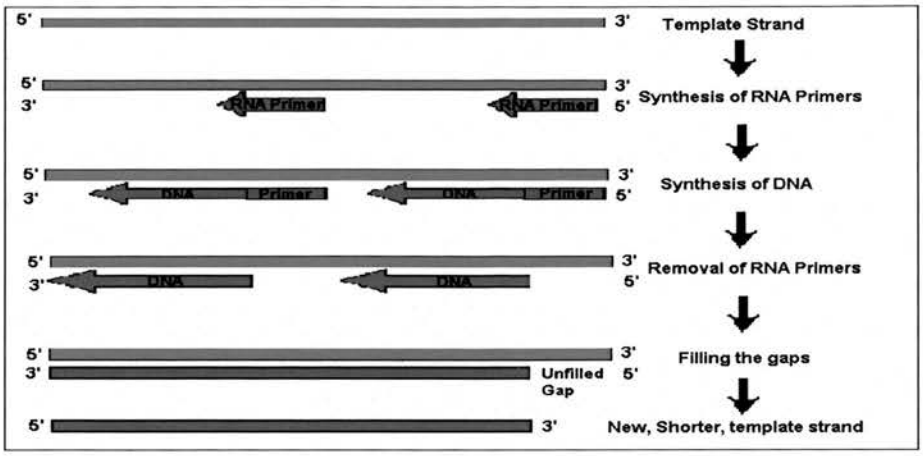


Figure 1.3.1: Schematic representation of the problem associated with completely replicating a linear chromosome. After semi conservative replication, the new strands have RNA primers at their 5' ends. RNA primers are removed and DNA polymerase fills in the resulting gaps and DNA ligase joins the adjacent fragments. However, at the 5' end of each new strand, gaps remain because DNA polymerase cannot initiate new DNA synthesis.

Telomerase is a ribonucleoprotein which functions, independently of DNA polymerase, to add telomeric repeat sequences (TTAGGG, in humans) to the ends of chromosomes, maintaining chromosome length and integrity (Greider & Blackburn, 1996). Telomerase is comprised of two components: human Telomerase RNA (hTER), an RNA element that serves as a template for the polymerase activity of the second component, telomerase reverse transcriptase (hTERT), which is responsible for the enzymatic activity of telomerase (Tzukerman *et al.,* 2000; Kyo *et al.,* 2000; Meyerson *et al.,* 1997). In order to reconstitute telomerase activity *in vitro*, both hTER and hTERT are necessary (Weinrich *et al.,* 1997).

Diploid human somatic cells do not express telomerase; consequently, telomeres shorten with age and cells enter replicative senescence after a finite proliferative life span in tissue culture (Allsopp *et al.,* 1992). The re-introduction of telomerase

activity has been demonstrated, in some human somatic cells, to extend their replicative life span (Bodnar *et al.,* 1998) suggesting a significant correlation between telomerase expression and immortality in human cell lines. Conversely, telomerase activity has been readily detected at the earliest gestational stages during human embryonic development (Wright *et al.,* 1996) and in accordance with their origin, human ES cells have also been observed to be strongly telomerase positive (Reubinoff *et al.,* 2000; Tzukerman *et al.,* 2000; Thomson *et al.,* 1998). Furthermore as seen in the developing foetus (Wright *et al.,* 1996), a time-dependent decline of telomerase activity was reported following induction of differentiation (Tzukerman *et al,* 2000). The authors reported disappearance of telomerase activity after 14 days of differentiation, which they correlated with a marked reduction in hTERT promoter activity (Tzukerman *et al,* 2000).

Down regulation of the hTERT promoter following onset of differentiation in human ES cells, reported by Tzukerman *et al,* (2000), made this promoter an ideal candidate for use in the selective elimination strategies reported in this thesis. In addition to its regulated expression in undifferentiated ES cells, Tzukerman *et al,* (2000) also demonstrated that activation of telomerase is a major mechanism in the molecular pathogenesis of most, but not all, malignant tumours. Up-regulation of hTERT in 80-90% of the malignancies (Kim *et al.,* 1994) provides a potential way of controlling cells that revert back to an undifferentiated state or become neoplastic after transplantation of therapeutic cells *in vivo*.

Octamer Binding Protein 4 (Oct-4)

The Oct-4 gene encodes a transcription factor belonging to the POU (Pit, Oct, Unc) family of domain transcription factors. The POU domain is a bipartite DNA-binding domain which, in the case of Oct-4, binds to an octamer sequence motif present in the promoter or enhancer regions of target genes, thereby regulating their expression (Nordhoff *et al.,* 2001; Hansis *et al.,* 2000; Nichols *et al.,* 1998). Oct-4 expression has been reported to be specific to ES cells, preimplantation embryos, epiblasts and germ cells (Okamoto *et al.,* 1990; Scholer *et al.,* 1989). In mice, Oct-4 expression has been demonstrated to be essential in the zygote for establishment of the pluripotent stem cell population in the ICM (Nichols *et al.,* 1998). At gastrulation, Oct-4 expression is down-regulated in an anterior-posterior pattern. The only cells maintaining Oct-4 expression after this stage are primordial germ cells (PGC's), arising at day 7.2 of mouse development (reviewed by Pesce & Scholer, 2001). Expression of Oct-4 is maintained in female germ cells until the initiation of sexual differentiation of the gametes and meiosis at 13-14dpc and until the beginning of spermatogenesis in the newborn male (reviewed by Pesce & Scholer, 2001).

Oct-4 is one of the key regulators of pluripotent and germ line cells, maintaining them in the cycle of totipotency. Mouse embryos and ES cells that have an inactive Oct-4 gene lose their pluripotency and spontaneously differentiate into trophoblast lineages (Niwa *et al.,* 2000). However, over-expression of Oct-4 is unable to maintain ES cells in a state of self-renewal; in fact a two-fold increase in Oct-4 was sufficient to induce differentiation into primitive endoderm and mesoderm, while repression of Oct-4 leads to loss of pluripotency and dedifferentiation to

trophoectoderm (Niwa *et al.,* 2000). Thus it is the absolute level of Oct-4 that controls cell fate.

In addition to murine ES cells, Oct-4 mRNA has also been reported in human EC cells (Schoorlemmer *et al.,* 1995) and undifferentiated ES cells of non-human primates (Mitalipov *et al.,* 2003) and humans (Reubinoff *et al.,* 2000). Sequence comparison of the promoter/enhancer regions of the human Oct-4 gene with that of the mouse ortholog revealed a common organisation of *cis*-regulatory elements (Nordhoff *et al.,* 2001). Since the expression pattern of human Oct-4 was comparable to that of the mouse it was suggested that Oct-4 might have a similar function in preventing human totipotent embryo cells from differentiating (Hansis *et al.,* 2000). In accordance with this hypothesis, as in murine EC and ES cells (Wang & Schultz, 1996; Rosner *et al.,* 1990) Oct-4 expression has been shown to be down-regulated in primate ES cells, following differentiation (Mitalipov *et al.,* 2003; Reubinoff *et al.,* 2000; Kraft *et al.,* 1996; Schoorlemmer *et al.,* 1995) and inactivation of the Oct-4 gene, through RNA interference for example, leads to spontaneous differentiation of ES cells (Zaehres *et al.,* 2005; Matin *et al.,* 2004; Hay *et al.,* 2004).

Taken together, these data show that Oct-4 is not expressed in terminally differentiated cells but is highly expressed in pluripotent cells, making the Oct-4 promoter another candidate, in this project, for restricting the expression of desired cell surface marker genes, to undifferentiated human ES cells.

Nanog a homeoprotein capable of maintaining self-renewal

Initially, the need for the addition of LIF to maintain murine ES cells in their undifferentiated state led researchers to believe that it and the LIF receptor/gp130/STAT3 pathway were fundamental for the maintenance of self-renewal. However, a number of mutant mice, deficient in components of the LIF pathway, formed normal ICMs (Nichols *et al.,* 2001) and furthermore, self-renewal of human ES cells have been shown to be LIF independent (Amit *et al.,* 2000; Reubinoff *et al.,* 2000) suggesting that LIF is not fundamental for all self-renewal. This theory was confirmed with the identification and characterisation of the homeoprotein Nanog, which was able to maintain self-renewal in murine ES cells independently of the LIF/gp130/STAT3 pathway (Chambers *et al.,* 2003; Mitsui *et al.,* 2003). Nanog mRNA has since been found in EG cells of mouse and in human EC and ES cells (Zaehres *et al.,* 2005; Ginis *et al.,* 2004; Chambers *et al.,* 2003). Constitutive expression of Nanog, maintains murine ES cells in an undifferentiated state in the absence of LIF, while knockout and knockdown studies have shown that both mouse and human ES cells spontaneously differentiate without Nanog in conditions that would otherwise support undifferentiated growth (Zaehres *et al.,* 2005; Chambers *et al.,* 2003; Mitsui *et al.,* 2003). Furthermore, when RNA interference was used to knockdown either Oct-4 or Nanog separately in human ES cells, a corresponding down-regulation of the opposite protein was observed, indicating a reciprocal co-ordination of the expression of Oct-4 and Nanog (Zaehres *et al.,* 2005). The restricted expression of Nanog provides another potential candidate to provide ES restricted expression of transgenes.

Differences in characteristic marker expression between Mouse and Human EC and ES cells

As with telomerase, Oct-4 and Nanog, restricted expression of the zinc finger protein, Rex1 (Rogers *et al.,* 1991) and the transcription factor, Sox2 (Yuan *et al.,* 1995) to pluripotent stem cells and preimplantation embryos, have also been described in human ES cells (reviewed by Ginis *et al.,* 2004). However, expression of cell surface carbohydrate stage specific embryonic antigens (SSEA-) -1, -3 and -4 differ between murine EC and ES cells and their primate counterparts. Human EC cells and all primate ES cells have been shown to express high levels of SSEA-3 and -4, as well as tumour recognition/rejection antigens (TRA) -1-60 and -1-80, when in their undifferentiated state (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998; Thomson *et al.,* 1996; Thomson *et al.,* 1995; Andrews, *et al.,* 1985). Upon differentiation of human EC and ES cells, expression of these cell surface proteins is rapidly down-regulated while expression of SSEA-1 is transiently up regulated (Draper *et al.,* 2002). By contrast, mouse EC and ES cells do not express TRA-1-60 or TRA-1-81 and expression of the SSEA markers has the reverse pattern to human EC and ES cells, with positive SSEA-1 expression while undifferentiated and up regulation of SSEA-4 upon initiation of differentiation. Despite these differences in cell surface markers between ES cells from human and mouse, human ES cells have been shown to have cell surface markers characteristic of the developing human embryo, implying that the observed differences are probably species related (Henderson *et al.,* 2002). Although interestingly, undifferentiated human EG cells displaying positive expression of TRA-1-60, TRA-1-81, SSEA-1 and -4, but are negative for SSEA-3 (Pan *et al.,* 2005; Park *et al.,* 2003; Shamblott, *et al.,* 1998).

Evidence for the restricted expression of genes, such as telomerase, Oct-4 and Rex-1 etc., to preimplantation embryos, germ cells and pluripotent ES cells, provides an opportunity to use the promoters of these genes to drive lineage specific expression of reporter genes, which can then be used to identify undifferentiated ES cells *in vitro* (Gerrard *et al.,* 2005; Eiges *et al.,* 2001). Furthermore, the presence of endogenous cell surface epitopes that are naturally down-regulated upon differentiation provides an opportunity to assess the ability of these epitopes to identify undifferentiated human ES cells from a mixed population. This thesis will explore the potential of such ES specific characteristics to selectively eliminate undifferentiated human ES cells from within a mixed population as a method of removing the tumorigenic risk associated with ES-derived therapy.

1.3.5.3 Genetic Manipulation of Human ES Cells

Although there is a risk of malignancy associated with human ES cells, their indefinite growth in culture provides a unique opportunity to manipulate to some extent their fate, which means they continue to be a useful tool for the development of regenerative medicine. In 2001, Eiges *et al.* reported the first transgenic human ES cells, which carried the green fluorescent protein (GFP) gene under the transcriptional control of the Rex1 promoter (Eiges *et al.,* 2001). In this paper the authors described a comparison of a number of different techniques for delivering foreign DNA into ES cells, and assessed their efficiencies. By contrast to mice, where electroporation was found to be the method of choice for DNA delivery into

ES cells, Eiges *et al.,* (2001) reported that the most efficient method in human ES cells was the polyethylenimine, ExGen 500. Consequently, there are a number of reports in the literature documenting the use of Exgene 500 for the transfection of human ES cells, however the majority of these reports originate from the Benvenisty Laboratory (Urbach *et al.,* 2004; Lavon *et al.,* 2004; Schudiner *et al.,* 2003; Eiges *et al.,* 2001), with other researchers choosing cationic lipids such as FuGene 6 and Lipofectamine 2000, untested by Eiges *et al.,* (2001), as their method of choice to genetically modify human ES cells (Hay *et al.,* 2004; Park *et al.,* 2003; Zwaka & Thomson, 2003; Lebkowski *et al.,* 2001).

The use of cationic reagents for the transfection of human ES cells significantly increased cell survival compared to electroporation (personal observation). However, a disadvantage of these techniques was the high incidence of multicopy arrays at the site of integration (Pells S., and Priddle H., unpublished data). There are a number of reports, especially in plants and *Drosophila*, which demonstrate gene silencing from a transgenic locus when repeat copies of a transgene are arranged as a concatameric array (reviewed by Henikoff, 1998). It was hypothesised that reconfiguration of heterochromatin, initiated by the integration/repositioning of multiple copy arrays of euchromatic genes near to a region of heterochromatin, was responsible for the mosaic gene expression pattern, often referred to as position-effect variegation (PEV) (reviewed by Weiler & Wakimoto 1995).

To determine whether the same was true in the mammalian system, Garrick *et al.,* (1998), developed a strategy to test this hypothesis. Using the Lox/Cre system of

site-specific recombination the authors generated transgenic mice containing different copy numbers (>100, 5 and 1 copy) of a β-galactosidase transgene. This allowed specific analysis of variations in β-galactosidase expression as a direct result of copy number by eliminating variation as a result of position effects. The report clearly demonstrated that a reduction in copy number significantly increased transgene expression (Garrick *et al.,* 1998). In accordance with this work, McBurney *et al.,* (2002) also described evidence of repeat induced gene silencing (RIGS) in murine EC cells, however, in addition to RIGS, this report demonstrated that gene silencing of a single transgene copy also occurred with time in culture. The authors described how the addition of inhibitors of histone deacetylase temporarily prevented gene silencing, until they were removed, suggesting that there is an alternative method to RIGS by which transgenes are silenced (McBurney *et al.,* 2002).

The effects of PEV, often associated with random integration of transgenes, can be successfully overcome, through the use of gene targeting. The inclusion of sequence homology in the transgenic construct can be used to target the integration of a transgene into a specific locus, possibly pre-characterised to have stable expression. In 2003, Zwaka and Thomson reported the first example of homologous recombination in human ES cells. In this report the authors indicated that chemical transfection reagents (ExGen 500 and FuGene 6) were unsuccessful for homologous recombination and reported the first successful application of electroporation for human ES cells (Zwaka & Thomson, 2003). However, in 2004, Benvenisty's group reported the production of a model of Lesch-Nyhan Disease, through gene targeting of male human ES cells at the HPRT locus, using ExGene 500 (Urbach *et al.,* 2004).

The disadvantage of gene targeting, over the use of random integration of transgenes, is the frequency at which homologous recombination events take place. In the two reports mentioned above absolute targeting efficiencies ranged from as low as $0.2x10^{-7}$ using ExGene 500 (Urbach *et al.,* 2004) to the maximum efficiency of $4.0x10^{-7}$ following electroporation (Zwaka & Thomson, 2003), compared to a random transfection efficiency of between $1x10^{-6}$ and $2.3x10^{-5}$ respectively.

Another strategy to overcome PEV that has received significant interest over recent years is the use of viral transduction, in particular the use of lentiviral vectors (Martin *et al.,* 2005; Gropp *et al.,* 2003; Ma *et al.,* 2003) or adenoviral (Ad) or adeno-associated viral (AAV) vectors (Smith-Arica *et al.,* 2003). However, although viral transduction has been reported to be both highly efficient and stable (Martin et al., 2005; Gropp et al., 2003; Ma et al., 2003), there are a number of issues which hamper its routine use; 1) their limited capacity for DNA content, approximately 8kb for lentivirus and only 4.5kb for AAV, 2) that viral methods may induce immune responses and engender the possibility of recombination/mutation between vector and recipient DNA *in vivo* and 3) the associated need for complex preparations of the viral vectors and designated work areas to ensure their safe use.

1.4 The Risk of Tumorigenesis

The tumorigenic potential of undifferentiated ES cells is known to be significant following subcutaneous injection into SCID mice resulting in the formation of teratomas (Reubinoff *et al,* 2000; Thomson *et al,* 1998; Thomson *et al.,* 1995; Evans & Kaufman, 1981). However, *in vitro* differentiation prior to transplantation reduces this risk and there are now a number of reports showing successful engraftment of ES-cell derivatives, in the absence of teratoma formation (Keirstead *et al.,* 2005; Abraham *et al.,* 2004; Li *et al.,* 2003; Kim *et al.,* 2002; Zhang *et al.,* 2001; Reubinoff *et al.,* 2001; Brüstle *et al.,* 1999; McDonald *et al.,* 1999). Interestingly, all of these reports share a common factor: the transplants are all xenogenic, either mouse to rat or chicken, or human to rat or mouse. Homologous, mouse-to-mouse, engraftments have been reported in the literature as being successful. Lumelsky *et al.* (2001) reported survival and positive staining for insulin from ES-cell derived islet-like cells in diabetic mice for up to 6-weeks post transplantation; Klug *et al.* (1996) reported stable intra-cardiac grafts from differentiated ES-cells in adult dystrophic mdx mice for up to 7-weeks and Perlingeiro *et al.,* (2001), reported contribution of ES-derived haematopoietic cells to the bone marrow, peripheral blood and spleen when injected into irradiated mice. Unfortunately, however, these reports did not discuss the presence or absence of teratomas, and there is a growing body of evidence that has demonstrated the occurrence of teratomas at high frequency when homologous transplants of ES-derived cells are used (Asano *et al.,* 2003; Erdo *et al.,* 2003; Wakitani *et al.,* 2003).

In 2002, Hoehn *et al.,* demonstrated that undifferentiated murine ES cells transplanted into the brains of rats, with an experimental induced stroke injury, could migrate to a site of injury and undergo appropriate differentiation with no evidence of teratomas, an observation that has been reported by others (Park *et al.,* 2003; Björklund *et al.,* 2002; Hoehn *et al.,* 2002). The authors hypothesised that the absence of teratomas in that model was perhaps not related to the differentiation status of the ES cells, but that it reflected a tumour suppressive effect of the xenogenic host tissue (Hoehn *et al.,* 2002). In successive work, the group compared the incidence of teratoma formation in transplantation experiments with murine ES cells into the xenogenic rat model and into a homologous mouse model of stroke (Erdo *et al.,* 2003). The authors reported that despite the use of undifferentiated ES cells, tumorigenesis in the rat was rare, with only 2 out of 22 rat brains displaying small microscopically visible tumours near the site of implantation. By contrast, 10 out of the 11 mouse brains exhibited large, macroscopic tumours that displayed characteristics of malignant teratocarcinomas (Erdo *et al.,* 2003). Xenogenic transplants of human ES cells into SCID mice result in the formation of teratomas, and are currently used to prove that human ES cells have the potential to differentiate into cell types representative of all three germ layers, *in vivo*. This indicates that not all xenogenic transplants are protected from tumour formation, suggesting that perhaps mice are more prone to tumours that other animals. To test this, rat ES cells would need to be transplanted as homologous and xenogenic grafts and the incidence of tumour formation assessed.

Interestingly, the report by Erdo *et al.,* (2003) also indicated that tumorigenesis was induced by as few as 500 undifferentiated (Oct-4 positive) murine ES cells irrespective of the site of implantation. Furthermore, pre-differentiation of murine ES cells into neural progenitors, containing less than 0.5% Oct-4 positive cells still resulted in 86% of the mice developing tumours within 2-weeks of transplantation into the brain. The report went on to show that within the tumour tissue, the number of Oct-4 positive cells had increased, implying that undifferentiated cells proliferated prior to tumour formation (Erdo *et al.,* 2003).

Whether rats have tumour suppressive properties or mice have an increased risk of tumour formation, any potential for tumour development from therapeutic ES cell therapy must be addressed and evaluated before any considerations of clinical use can be pursued.

1.5 Strategies to Eliminate Potentially Tumorigenic Cells

Recently, two groups have reported selective ablation strategies for ES cells, Fareed and Moolten (2002) in mouse and Schuldiner *et al.* (2003) in human ES cells. Both of these strategies are based upon a toxic gene-therapy approach: the introduction of the herpes simplex virus thymidine kinase (HSV-tk) suicide gene.

In general a suicide gene encodes an enzyme that is not normally present in mammalian cells, that converts an inactive prodrug into a toxic product, conferring sensitivity to that drug. This technique was initially used, with great success, as an inducible toxin for studying loss of cell function in animal models, since it overcame the issue of embryo lethality associated with cell function knockouts (Heyman *et al.*, 1989; Borrelli *et al.*, 1988). HSV-tk is the most extensively studied suicide gene and has been used in cancer elimination strategies with varying degrees of success (Kuriyama *et al.*, 1996; Beck *et al.*, 1995; Golumbek *et al.*, 1992; Culver *et al.*, 1992). The HSV-tk enzyme efficiently phosphorylates the harmless prodrug ganciclovir (GCV) into a monophosphorylated molecule, which is subsequently converted by cellular phosphokinases into the toxic triphosphate substrate. The triphosphate form of GCV is then incorporated into elongating DNA during cellular division resulting in inhibition of DNA synthesis through chain termination, therefore, selectively killing dividing cells (Kuriyama *et al.*, 1996; Mar *et al.*, 1985). Normal mammalian cells are protected from the inactive drug, since GCV is a relatively poor substrate for their cellular thymidine kinases. Consequently, concentrations of GCV that are lethal to HSV-tk expressing cells are non-toxic to wild type cells (Borrelli *et al.*, 1988).

The transfer of GCV mediated inhibition of DNA synthesis was first reported by Culver *et al.,* (1992) between HSV-tk$^+$ and HSV-tk$^-$ tumour cells (now referred to as the bystander effect). Since then it has been shown that efficient tumour reduction can be achieved whether the population contains 10% or 100% HSV-tk transduced cells (Wu *et al.,* 1994). However, the bystander effect has only been observed when co-cultures of positive and negative cells were grown at high density and it was suggested that transfer of the toxic effects of GCV occurred through physical contact, probably through gap junctions (Wu *et al.,* 1994; Bi *et al.,* 1993). Furthermore, transfer of phosphorylated GCV has been shown not to be restricted to neighbouring cells of the same cell type or even of the same species, increasing the potential of this strategy for use in the treatment of cancers (Chen *et al.,* 1995; Wu *et al.,* 1994).

The reports of Fareed and Moolten (2002) and Schuldiner *et al.,* (2003) describe the use of HSV-tk as a possible fail-safe system against immune pathology, hyperfunction or the emergence of malignancies as a result of ES-derived grafts. Fareed and Moolten (2002) demonstrated that undifferentiated mouse ES cells are highly sensitised to GCV by HSV-tk transduction and that they retain sensitivity, although to a lesser extent requiring increased concentrations of GCV, when differentiated along haematopoietic lineages. Consequently, with slightly elevated concentrations of GCV, still within those approved by the regulatory bodies, ES-derived grafts, which have developed post-transplantation side effects, could be selectively removed. In addition, the authors reported that HSV-tk negative murine ES cells were sensitive to clinically applied levels of GCV in the absence of co-

culture, suggesting that if gene expression was lost *in vivo*, the ability to eliminate undifferentiated ES cells could be maintained using this technique (Fareed & Moolten, 2002).

In a similar strategy, Schuldiner *et al.,* (2003) recently reported that undifferentiated human ES cells, which constitutively expressed HSV-tk, were specifically eliminated *in vitro* and *in vivo* when GCV was administered. Established tumours were significantly reduced in size and lost growth potential as a result of GCV administration, with no evidence of toxicity to untransduced cells (Schuldiner *et al.,* 2003). However, the report provided no data to support sensitivity or protection of differentiated derivatives of these HSV-tk expressing human ES cells and so it is unknown as to whether, as shown for mice, increased concentrations of GCV would be required to completely eliminate a therapeutic graft, if the need should arise.

The reports of both Fareed and Moolten, (2002) and Schuldiner *et al.,* (2003) demonstrate the lack of a "bystander effect" between ES cells. If this were true for differentiated derivatives of ES cells, then following administration of GCV areas surrounding the graft would be protected from collateral damage. Alternatively, if ES cell derivatives do demonstrate a "bystander effect", then should GCV be administered to control tumour growth this could lead not only to complete loss of the therapeutic graft, resulting in onset of disease, which would require further intervention, but could potentially cause further damage to the surrounding tissue.

The strategy reported in this thesis utilises restricted expression (see section 1.3.5.2) of a foreign cell surface epitope, which leads to exclusive expression on undifferentiated, potentially tumorigenic, human ES cells. This epitope can then be used to specifically eliminate undifferentiated human ES cells from a mixed population *in vitro*, or *in vivo* through either an innate or acquired form of immunity i.e. vaccination against the foreign epitope.

The advantage of this strategy is that when the elimination strategy is applied *in vitro,* functional ES-derived therapeutic cells will be protected from its action, unlike those reported in the HSV-tk strategy, which have constitutive expression of the suicide gene, this reduces the risk of inadvertently transplanting potentially tumorigenic cells. If part of an ES-derived cell graft led to post-transplantation malignancy *in vivo*, using the strategy reported in this thesis, only the malignant part of the graft would be removed, leaving behind the rest of the graft to function appropriately. However, with the HSV-tk strategy, elimination of the whole graft would follow treatment with GCV, which would require further intervention to prevent onset of disease symptoms.

Immunisation against the foreign epitope has the possibility to provided *in vivo* surveillance for undifferentiated, dedifferentiated or malignant cells, which either evaded *in vitro* elimination or occurred post-transplant. The advantage of this over treatment with GCV is obviously the time at which it occurs. Immune surveillance will be able to tackle emerging malignancies before they become significant or are clinically detectable, which is when GCV administration would start.

However, exclusive expression of foreign epitopes to undifferentiated cells is a strategy that will only eliminate potentially tumorigenic cells, it will not be possible to remove grafts with hyperfunction or immune pathology using this technique. It is likely that in the future, combination strategies will need to be developed; with one elimination strategy that is targeted to removing potentially tumorigenic cells, while the other strategy is constitutive to the whole graft.

1.6 Candidate Identification Markers

The strategy used in this project relies upon efficient identification and elimination of contaminating undifferentiated ES cells from a mixed population. To achieve this the intention was to express, under the transcriptional control of either the hTERT or Oct-4 promoter (Section 1.3.5.2), a cell surface marker of foreign origin that could be used to induce either an *in vitro* or *in vivo* immune response or which could be used in cell sorting techniques.

1.6.1 H2-K^k – a murine major histocompatibility complex antigen

In the mouse the classical class I major histocompatibility (MHC) antigens, or H-2 antigens, are encoded at three loci: K, D and L (Drezen *et al.,* 1993; Warner & Gollnick, 1993). The primary immune function of MHC class I molecules is to present peptides to CD8+ cytotoxic T-cells allowing them to recognise foreign molecules in the context of self. In addition, MHC class I molecules are also responsible for the protection of self-cells from lysis by natural killer (NK) cells (Drezen *et al.,* 1993).

H-2 is expressed on virtually all mouse tissues; however, there is a level of cell-specific regulation, with the highest level of expression being observed in lymphoid organs and the liver, and low expression seen in testis and brain (Drezen *et al.,* 1993). Furthermore, H-2 expression is developmentally regulated. Initially, a degree of controversy surrounded expression of MHC class I on preimplantation embryos, but with the increased sensitivity of quantitative RT-PCR and RIA's (Radio-

Immuno-Assay) this has since been confirmed (Warner & Gollnick, 1993). Active H-2 mRNA transcript has been detected as early as the two-cell stage of development (Jin *et al.,* 2002); however, H-2 cell-surface expression was detectable only at day 10 of gestation, using RIA (Ozato *et al.,* 1985). H-2 mRNA expression increases dramatically between day 11 and day 13 post coitum, and progressively thereafter, reaching adult levels at around six-weeks postpartum (Warner & Gollnick, 1993).

MHC has been the subject of intense study over the years, and consequently there are a number of commercial products available in this area. *Miltenyi Biotec Ltd.* supplies a plasmid that contains a truncated version of an H2-K alloantigen, H2-K^k, called *pMACS* K^k*.II,* and also a selection of IgG_2 antibodies against H2-K^k that could be employed in this investigation. Endogenous expression of H2-K^k is restricted to a limited number of mouse strains, including AKR/J, CBA/Ca CBA/J, CBA/N, C3H/Bi and C3H/He, which will be used as positive controls. Strains BALB/cJ or BALB/cAnN and 129/sv express MHC I of a different haplotype and will be used as negative controls.

1.6.2 The α-galactosyl (α-gal) epitope and anti-α-gal antibodies

The α-galactosyl epitope (Galα1-3Galβ1-4GlcNAc-R termed α-gal) is an abundant (>10^6 epitopes/cell) carbohydrate structure present on the cell surface of all non-primate mammals, prosimians and South American monkeys (Galili *et al.,* 1988a; Galili *et al.,* 1987a). Its synthesis is catalysed by the glycosylation enzyme α1,3galactosyltransferase (α1,3Gal) within the trans-Golgi network. The activity of

α1,3Gal is to transfers galactose from uridine diphosphate galactose (UDP-Gal) to the N-acetyl-lactosamine acceptors on carbohydrate side chains of glycoproteins and glycolipids creating the α-gal epitope:

(Galβ1-4GlcNAc-R+UDP-gal $\xrightarrow{\text{α1,3galactosyltransferase}}$ Galα1-3Galβ1-4GlcNAc-R+UDP).

The activity of α1,3Gal was first described in rabbit bone marrow by Basu and Basu (1973) and has since been observed in non-primate mammals, prosimians and New World monkeys (NWM), but not in monkeys of the Old World (OWM), apes or humans (Galili *et al.,* 1988a). The loss of α1,3Gal activity in humans has been attributed to two prominent mutations within the gene, which have resulted in a frame shift, leading to a premature stop codon and consequently a truncated protein (Galili & Swanson, 1991; Larsen *et al.,* 1990). Using comparative genome studies, DNA from species of hominoids (apes), gorilla, orangutan, OWM, human, NWM and non-primate mammals has been assessed for the degree of acquired mutations in the α1,3Gal gene over time (Galili & Swanson, 1991). These studies have indicated that α1,3Gal gene suppression was constrained to the Old World, but occurred independently in apes and monkeys following their divergence less than 28 million years ago (Galili & Swanson, 1991).

It has been hypothesised that α1,3Gal gene suppression in ancestral primates resulted from an extreme selection pressure, probably caused by a pathogen, confined to the Old World which led Old World primates into near extinction (Galili & Swanson, 1991; Galili *et al.,* 1988a). In support of this hypothesis, expression of α-gal epitopes has been found on numerous strains of virus, bacteria and some protozoa (Rother &

Galili, 1999; Galili *et al.,* 1988b; Avila 1999) and in addition there is also evidence to suggest that toxins, such as enterotoxin A of *Clostridium difficile*, use cell surface α-gal epitopes as receptors for their activity (Phelps *et al.,* 2003; Clark *et al.,* 1987). There are reports of analogous examples of pathogens that have shaped the evolution of Old World primates, such as Epstein Barr virus (EBV) and geographical variation in the resistance of humans to pathogens such as the Smallpox virus (reviewed by Galili, 1999a).

Whatever the evolutionary event that was responsible for the fixation of mutations within the α1,3Gal gene in ancestral primates, it is likely that the transition was not a gradual process. Fossil evidence from the early Miocene (17-22 million years ago) shows that there were significantly more species of apes than at the present time, and that the relative number of individual ape fossils discovered from that period was high. Conversely after this period, ape fossils began to disappear, becoming very rare after 7-8 million years ago. It has been speculated that this massive extinction in the ape population could reflect the event that led to α1,3Gal suppression (reviewed by Galili, 1999a).

Apes, humans and OWM have been shown to produce a high titre of natural anti-α-gal antibody, which is not present in the sera of non-primate mammals and NWM, the reciprocal of the α1,3Gal gene expression pattern (Galili *et al.,* 1987a; Galili *et al.,* 1984). Anti-α-gal is the most abundant natural antibody found in humans, the IgG form of this polyclonal antibody was found at serum concentrations of between 30-70μg/ml, constituting 1% of total circulating IgG (Galili *et al.,* 1984, 1985).

Furthermore, with the exception of a drop in production between ages 3-6 months, which corresponds to a decrease in total maternal IgG and the initiation of self-IgG synthesis, production of anti-α-gal remains constant throughout life reaching adult levels by age 2-4 years (Wang et al., 1995; Galili et al., 1984). The continuous production of anti-α-gal throughout life suggested that chronic antigenic stimulation was responsible for the maintenance of such high antibody titre. In the 1970's it was shown that natural anti-blood-group antibodies interacted with carbohydrate structures shared between the normal gastrointestinal bacteria and human blood-group antigens, and it was suggested that this stimulus was responsible for the constant production of anti-blood group antibodies (Springer *et al.,* 1971). Through direct immunostaining and ELISA, anti-α-gal was also found to readily bind to a variety of *E. coli*, *Klebsiella*, *Serratia* and *Salmonella* isolates from normal stool samples, suggesting that gastrointestinal bacteria also provide antigenic stimulation for the production of anti-α-gal (Galili *et al.,* 1988b). However, although the production of anti-α-gal antibody remains constant, binding affinity between individuals varies significantly (as much as 25-fold), with a significant increase in the amount of low-affinity binding of anti-α-gal antibodies in the elderly, age 70-90 (Oostingh *et al.,* 2003; Wang *et al.,* 1995). The low binding affinity of anti-α-gal is thought to be due to a lack of ionic bonds between anti-α-gal and the α-gal epitope although the reason for increased low affinity antibody in the elderly remains unknown (Galili *et al.,* 1999b).

It has been shown that specificity of anti-α-gal IgG is dependent upon an individual's blood type. The structure of the blood group-B antigen and α-gal are similar and it

has been shown that less than 25% of anti-blood group-B antibodies from blood group-A and -O individuals are specific to the blood group-B antigen, the rest are cross-reactive anti-α-gal B antibodies (Galili *et al.,* 1987b). It has also been shown that there is slight cross-reactivity to the blood group-A antigen suggesting that sera from blood group-O individuals has the broadest spectrum of anti-α-gal antibody activity (Galili *et al.,* 1987b). As expected, individuals of blood group-AB or-B have the narrowest spectrum of anti-α-gal IgG activity, which is specific to the α-gal epitope; immune tolerance prevents the occurrence of B-lymphocyte clones that produce cross-reactive anti-α-gal B antibodies, as a mechanism against autoimmunity (Oostingh *et al.,* 2003; McMorrow *et al.,* 1997; Galili *et al.,* 1987b). It is unclear whether this blood group dependent difference in the spectrum of anti-α-gal IgG activity alters the overall titre of antibody able to bind α-gal in the event of immune challenge (Oostingh *et al.,* 2003; McMarrow *et al.,* 1997; Galili *et al.,* 1984). There was no evidence of blood group dependent IgM activity (Oostingh *et al.,* 2003; McMarrow *et al.,* 1997).

The interaction between anti-α-gal antibodies and the α-gal epitope is of major clinical significance, especially in the field of transplantation. The level of natural anti-α-gal antibody described thus far, indicates the level of anti-α-gal in response to a steady state of stimulation by normal gastrointestinal bacteria. However, when stimulated by the transplantation of non-primate organs or tissues (xenografts), the level of anti-α-gal IgG production, in particular, increases dramatically resulting in rapid rejection of the graft, a process termed hyper acute rejection (HAR).

There is significant evidence to support HAR as a result of α-gal; transplantation of pig islets to humans for example, increased anti-α-gal IgG production 20- to 200-fold (Galili *et al.,* 1995; Satake *et al.,* 1994), grafting of pig articular cartilage and meniscus tissue in cynomolgus monkeys increased anti-α-gal IgG 30- to 300-fold (Galili *et al.,* 1997), while intravenous infusion of pig bone marrow into baboons (Kozlowski *et al.,* 1998) and *ex vivo* perfusion of pig livers in humans (Cotterell *et al.,* 1995) increased anti-α-gal IgG by 200-fold and 60-fold after 7-21 and 10 days respectively. Importantly, the observed increases in anti-α-gal antibody production occurred despite heavy immunosuppression, suggesting that removal of anti-α-gal antibody from xenograft recipients prior to engraftment would be insufficient to protect the graft from rejection. Long-term suppression of anti-α-gal antibody production in graft recipients or suppression of α-gal expression on the donor graft would be necessary to prevent HAR (Yan *et al.,* 2003; Chung *et al.,* 2003; Sendai *et al.,* 2003; Phelps *et al.,* 2003; Dai *et al.,* 2002; Sepp *et al.,* 1999). However, both of these strategies could increase the risk to both the donor and/or the recipient through infection, since α-gal and anti-α-gal play an important role in protection against pathogen invasion (Kobayashi & Cooper, 1999).

Although α-gal expression on donor grafts and anti-α-gal antibody production in recipients is an obstacle for transplantation therapy, in terms of advances in the treatment of cancers, natural immunity to α-gal expression holds great promise. In 1998, Link *et al.,* reported for the first time that transduction of human cancer cells with retroviral vectors containing the α1,3Gal gene, resulting in greater than 90% specific lysis of infected cells by human serum, showing that pretreated cells failed

to establish tumours after transplantation into mice (Link *et al.,* 1998). Since then several other reports have shown that numerous human cancer cell lines can be efficiently eliminated by human serum following transfection/transduction with the α1,3Gal gene and their ability to establish tumours following treatment can be reduced (Aubert *et al.,* 2003; Unfer *et al.,* 2003; Sawada *et al.,* 2002; Yoshimura *et al.,* 2001; Jäger *et al.,* 1999). Furthermore, Yoshimura *et al.,* (2001) reported that lysis of α-gal expressing human pancreatic and hepatocellular carcinomas were efficient irrespective of the blood type of serum from which the anti-α-gal antibodies were obtained (Yoshimura *et al.,* 2001). In combination with the possibility of directed gene therapy of human tumours through the transduction of tumour cells with α1,3Gal, there is also the possibility of using α-gal as a method for improving tumour vaccination technology, by increasing the immunogenicity of tumour vaccinations. By immunizing α1,3Gal knockout mice with irradiated tumours cells that were engineered to express α-gal on their cell surface, LaTemple *et al.,* (1999), were able to show an improved protective immune response against the same tumour cells which lacked the α-gal epitope (LaTemple *et al.,* 1999). Expression of α-gal on the surface of irradiated tumour cells acted as an opsonin, encouraging phagocytosis by antigen presenting cells, which in turn led to the presentation of tumour associated antigens and the stimulation of T-cells towards tumour cells that didn't express α-gal (LaTemple *et al.,* 1999).

The use of α-gal as a mechanism to initiate cell death directly in transfected cells or to indirectly target cells through immunization, provides evidence that the response

to the presence of α-gal epitopes is not restricted to the endothelium of vascularised tissues and organs, as is the case in HAR.

1.6.3 Cell Surface epitopes endogenous to Human ES cells.

As described in section 1.3.5.2, there are endogenous cell surface epitopes that are used to characterise undifferentiated human ES cells, namely the stage specific embryonic antigens-3 and -4 and the tumour recognition/rejection antigens-1-60 and -1-81.

1.6.3.1 Stage Specific Embryonic Antigens (SSEA-)

Stage specific embryonic antigens (SSEA-) are cell surface markers which are developmentally regulated (reviewed by Fenderson *et al.,* 1990). SSEA-3 and -4 make up different portions of an extended globoseries oligosaccharide found in glycolipids and glycoproteins. They are expressed on human EC, ES and EG cells and on erythrocytes (Henderson *et al.,* 2002; Tippett *et al.,* 1986) as determined by antibody binding assays with the monoclonal antibodies MC631 and MC813-70 respectively. However, their expression is not necessary indicative of cells with undifferentiated growth potential. Human EC cells negative for expression of both SSEA-3 and -4 when transplanted into mice were shown to give rise to malignant teratocarcinomas. When placed back into culture, EC cells isolated from these tumours expressed both SSEA-3 and -4 (Andrews *et al.,* 1985). SSEA-1 is a fucosylated polylactosamine antigen, also referred to as Lewis X antigen (LeX), which has been found to be expressed on murine EC and ES cells (Muramatsu &

Muramatsu 2004; Fox *et al.,* 1981) on human EG cells (Shamblott *et al.,* 1998) and transiently on differentiating human ES cells (Draper *et al.,* 2002).

1.6.3.2 Tumour Recognition/Rejection Antigens (TRA-)

Tumour recognition/rejection antigens (TRA-) -1-60 and -1-81 were first described on the cell surface of human EC cells, and were used as a method of detecting and evaluating the progression of germ cell carcinomas (Marrink *et al.,* 1991; Andrews *et al.,* 1984). There are two commercially available antibodies Tra-1-60 and Tra-1-81 (Chemicon) which have been found to bind to different epitopes of a keratan sulphate proteoglycan on the surface of a number of tumour derived cell lines (Andrews *et al.,* 1996; Andrews *et al.,* 1984). There is little else known about these antigens, other than that they are also strongly expressed on human EG cells and undifferentiated ES cells from primates (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998; Thomson *et al.,* 1996; Thomson *et al.,* 1995) and that their expression is rapidly lost with differentiation (Draper *et al.,* 2002).

The expression patterns or SSEA- and TRA- make them ideal candidates for use in selective elimination strategies for the removal of contaminating undifferentiated ES cells from a differentiated population. This natural ability of SSEA- and TRA- will be compared to transgenic identification strategies utilising H2-K^k and α-gal.

1.7 Eliminating Specific Cell Populations

With the transcriptional regulation of the foreign epitope being controlled by either hTERT or Oct-4, and the expression of SSEA and TRA- being naturally restricted, expression should only be observed on undifferentiated human ES cells. Eliminating only these cells *in vitro* prior to transplantation would rely on methods of either specific cell killing or depletion of these cells through cell sorting, while *in vivo* elimination would rely upon activation of innate or acquired immunity.

1.7.1 Complement Mediated Cell Lysis

Complement is a major component of the innate immune system, involved in self-non-self recognition. It is composed of a series of circulating plasma proteins, distinct membrane-bound receptors and regulatory proteins that work together and with other cells of the immune system to eliminate foreign cells. Once activated, the complement cascade plays a pivotal role in the initiation of a number of immune responses including; chemotaxis of inflammatory cells, enhancement of phagocytosis by neutrophils and monocytes, facilitation in the clearance of immune complexes and mediation of cell lysis by the formation of a membrane attack complex (MAC) (reviewed by Walport, 2001).

1.7.1.1 Complement activation

Activation of complement can be achieved through one of three methods; the "Classical" antibody-antigen dependent pathway, the "alternative" pathway or the "lectin" pathway. Non-immune activators, such as susceptible foreign surfaces, i.e.

bacteria and yeast cell walls, initiate the alternative pathway (reviewed by Song *et al.,* 2000). Activation via this pathway is an immediate line of defence that requires no immunological memory and begins the complement cascade by direct cleavage of C3 (Lambris, 1990). Similarly, the lectin pathway is activated by mannose-binding lectin (MBL), a serum protein, which binds particular carbohydrates such as mannose or N-acetylglucosamine on the surface of microorganisms (Turner, 1996; Kawasaki *et al.,* 1983). While the alternative pathway directly activates C3, in the lectin pathway, after binding with sugars on bacterial surfaces, MBL associates with, and activates, specific serine proteases, MBL-associated serine protease (MASP), which then cleave C4 and C2, activating the classical pathway (1.7.1.2) (reviewed by Song *et al.,* 2000).

1.7.1.2 Complement Activation via the Classical Pathway

It is the binding of antibody to antigen that was used in this thesis as a method of initiating complement-mediated lysis. Antigen-bound antibody molecules trigger the classical pathway of complement activation, either as immunoglobulin (IgG or IgM) bound directly to a cell surface antigen or as an antigen-antibody immune complex. Inactive serum protein C1 interacts with the Fc portion of either two molecules of bound IgG or one molecule of bound IgM to form a recognition complex that initiates the complement cascade. It is only when the antibodies bind to their antigen that binding sites, known as antibody receptor sites, for C1q are uncovered.

IgG isotypes vary in their capacity to bind C1q, in humans IgG_3 has the greatest capacity followed by IgG_1 and IgG_2. IgG_4 cannot bind C1q and is not a complement-fixating antibody (Bindon *et al.,* 1988). Once bound, C1q undergoes a conformational change, which causes two C1r molecules to cleave and activate each other. Subsequently, these cleave and activate the C1s molecules, which will then activate the next two complement proteins, C4 and C2, (Figure 1.7.2).

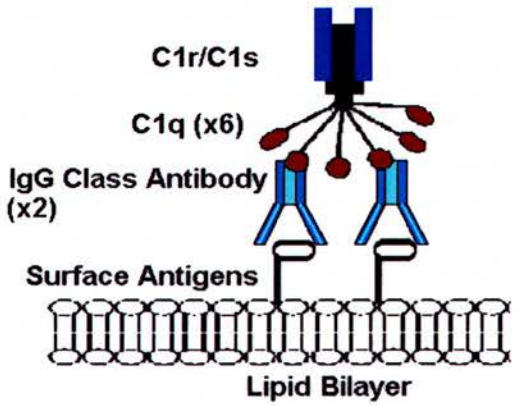


Figure 1.7.1: Schematic representation of the C1 Complement recognition complex. Binding of antibody to C1q activates C1r and C1s, which leads to cleavage of C4 and C2

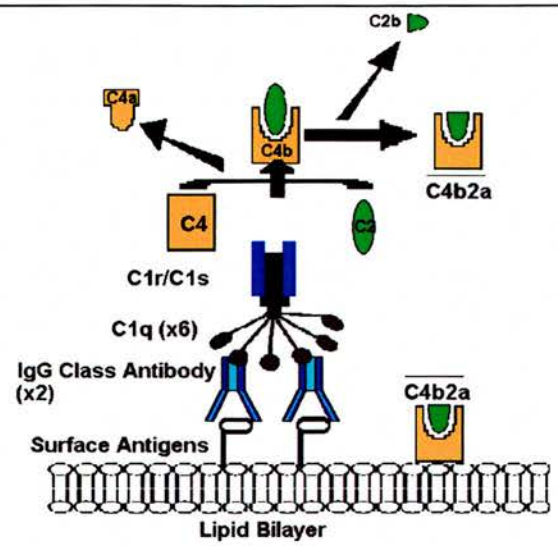


Figure 1.7.2: Schematic representation of the formation of C3 convertase. Activated C1s cleaves C4 into C4a and 4b. C4b binds C2, which is subsequently cleaved by C1s releasing the C2b fragment. The resulting C4b2a complex is capable of cleaving C3 and hence has been named C3 convertase

Activation of C4 and C2 involves the cleavage of a specific peptide bond within each molecule, which results in the dissociation of peptide fragments, C4a and C2b, and the exposure of a binding site in the larger fragments C4b and C2a. The C4b peptide binds to the cell membrane while C2a attaches to the C4b fragment forming the C4b2a complex. This complex is enzymatically active and is referred to as C3 convertase, since it binds and cleaves C3, the next inactive complement component. The C3 convertase is an unstable enzyme and undergoes a time and temperature-dependent decay, lasting only seconds, unless there is a sufficient quantity of C3 within close proximity of the cell-bound complex to mediate the next stage (reviewed by Goldsby *et al.,* 2000 & Roitt, 1997).

Activation of C3 results in the generation of a second convertase enzyme, C5 convertase, which occurs when C3 is cleaved by C3 convertase into C3a and C3b. The larger fragment C3b attaches both to the cell membrane and to the C3 convertase, creating a catalytic site to accommodate C5 and hence the complex is named C5 convertase (Figure 1.7.3).

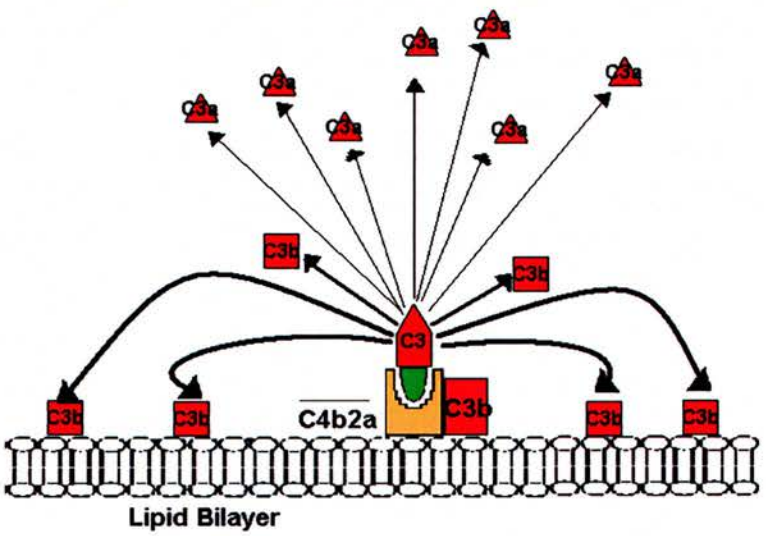


Figure 1.7.3: Schematic representation of the activation of C3: C3 is cleaved by C3 convertase into C3a and C3b fragments. C3a is released into the circulation where is it involved in the inflammatory response as an anaphylatoxin. C3b is either bound to the C3 convertase to form C5 convertase, or it is released, either to be bound directly by the cell membrane or to near by bacterium as a method of opsonization.

In addition to forming C5 convertase, C3b fragments can also be bound directly to the cell membrane, activating the alternative pathway or may diffuse away from the surface (but not more than 40nm) and coat immune complexes, opsonizing them for the phagocytes. C3 activation signifies an amplification step in the complement cascade, with a single C3 convertase molecule generating up to 200 molecules of

C3b, given the opportunity (Figure 1.7.3) (reviewed by Goldsby *et al.,* 2000 & Roitt, 1997).

Whichever initiation pathway activates the complement cascade, formation of C5 convertase signifies the beginning of the same terminal attack sequence (cell lysis), known as the membrane attack complex (MAC).

1.7.1.3 The Membrane Attack Complex

The membrane attack complex (MAC) is a multimolecular assembly of complement components C5-C9, which forms as a consequence of complement activation. Formation of the MAC complex begins with the cleavage of C5 by the C5 convertase (C4b2a3b), into C5a and C5b. C5a is released once formed into the circulation where is plays no further part in the complement cascade, instead it has an important role in inflammation, in the recruitment of phagocytes. C5b on the other hand, attaches to the cell membrane, uncovering binding sites for C6 and C7, producing a stable C5b67 complex attached to the cell membrane (Figure 1.7.4). C5b is a very labile molecule, if it remains unbound by C6 for more than 2 minutes it dissociates (reviewed by Goldsby *et al.,* 2000).

Binding of C7 to the C5b6 complex results in a hydrophilic-amphiphilic structural transition, generating hydrophobic binding regions for the membrane phospholipids. Consequently, the C5b67 complex anchors into the cell membrane, committing MAC assembly to that membrane site, forming receptors for C8. However, if the

reaction occurs on a non-cellular activating surface, then the hydrophobic sites cannot anchor the complex and it is released. Released C5b67 complex can insert themselves into the membrane of nearby cells, which if uncontrolled, could initiate an innocent bystander effect (reviewed by Goldsby *et al.,* 2000).

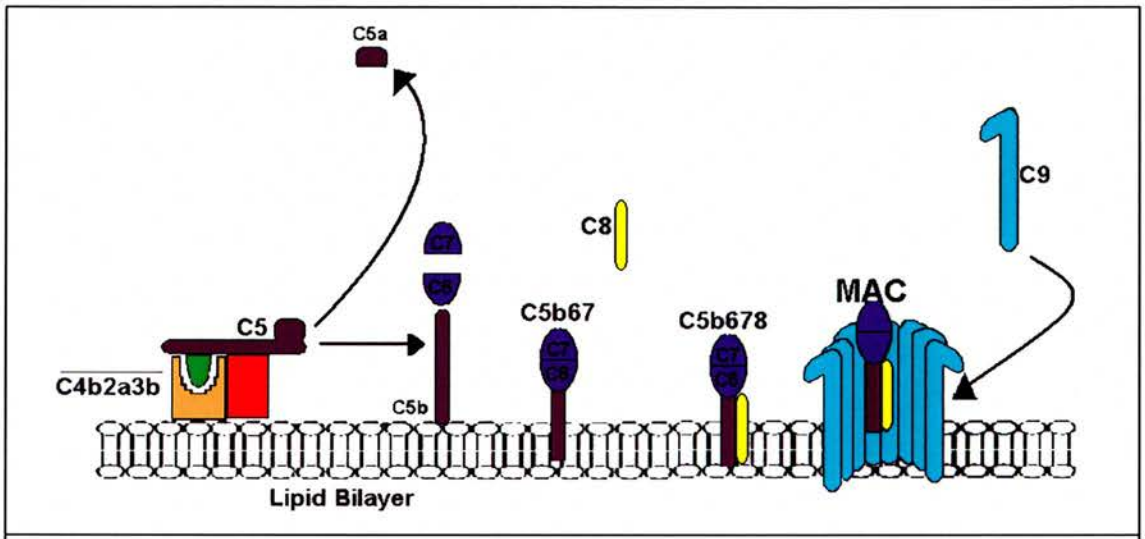


Figure 1.7.4.Schematic representation of the Membrane Attack Complex (MAC). Binding of C5b to the membrane acts as an anchor for C6 and C7, which encourages the binding of C8. C8 instigates the formation of small pores, but it is the binding of several C9 molecules which exaggerates the pores leading to membrane leakiness and death

Binding of C8 instigates the formation of small transmembrane channels of less than 1nm in functional diameter (Ramm et al 1982). These small pores cause the cells to become slightly leaky, and while they may be sufficient to cause lysis of red blood cells and nucleated cells (Martin *et al.,* 1987) killing of bacteria requires a more significant sized pore (Joiner *et al.,* 1985). Each membrane–bound C5b678 complex acts as a receptor for multiple C9 molecules. The recruitment of several C9 molecules initiates a substantial conformational change in these minor membrane attack complexes, from a globular, hydrophobic-form to an elongated, amphiphilic-

form, which spans 7-10nm in diameter, which transverse the membrane and exacerbates membrane leakiness, leading to cell death as a result of osmotic lysis (reviewed by Goldsby *et al.,* 2000).

1.7.1.4 Regulation of the Complement System

Although innate immunity is critical in providing a rapid first line of defence, it is important that it can still recognise self from non-self. Activated complement, if unregulated, can cause injury to host tissues through a bystander effect (Morgan, 1995). To prevent complement-mediated autologous attack, host tissues express a number of fluid-phase and membrane-bound regulators of complement (Table 1.7.1) (Hourcade *et al.,* 1989). The regulation of complement activity focuses on 2 key stages of the active cascade, the C3/C5 convertase and formation of MAC. Collectively these regulatory proteins ensure that inappropriate complement activation does not occur within normal host tissue.

There are three common membrane-bound proteins; membrane co-factor protein (MCP or CD46), decay-accelerating factor (DAF or CD55), and membrane inhibitor of reactive lysis (MIRL, most commonly known as CD59), which play an important role in regulating the complement cascade. The complement regulators DAF and MCP function to prevent assembly of the C3/C5 convertase enzymes. DAF is an effective decay accelerator in both the classical and alternative pathways and functions to block the formation of C3/C5 convertase by rapidly dissociating the enzymatic component C2a or Bb from membrane bound C4b or C3b respectively.

DAF has been reported to have a higher affinity for C4b and C3b when they are complexed with their respective catalytic subunits and therefore recycles from C4b and C3b sites to active C3 convertase enzymes (reviewed by Meri & Jarva, 1997). MCP on the other hand has no decay activity but blocks formation of C3/C5 convertase by binding to dissociated C4b or C3b preventing its association with the enzymatic component C2a or Bb and acting as an efficient cofactor for the serine protease factor-I, which irreversibly cleaves the C4b and C3b. Finally, CD59 protects cells from non-specific complement-mediated lysis by inhibiting formation of the MAC. CD59 binds to both C8 and C9 and thus prevents the assembly of the poly-C9 MAC and insertion into the cell membrane (Huang *et al.,* 2001; reviewed by Goldsby *et al.,* 2000; reviewed by Meri & Jarva, 1997).

Regulator	Type of protein	Immunologic function	Affected Pathway
C1 Inhibitor (C1-INH)	Soluble	Serine Protease inhibitor: causes dissociation $C1r_2s_2$ from C1q	Classical
C4b-binding protein (C4bBP)	Soluble	Binds to C4b preventing formation of C3 convertase	Classical and Lectin
Factor H	Soluble	Binds to C3b preventing formation of C3 convertase	Alternative
Complement Receptor Type-1 (CR1)	Membrane bound	Prevents the formation of C3 convertase by binding C4b or C3b	Classical, Alternative and Lectin
Membrane Co-factor protein (MCP, CD46)			
Decay-accelerating Factor (DAF, CD55)	Membrane bound	Accelerates the decay of C3 convertases (C4b2a and C3bBb)	Classical, Alternative and Lectin
Factor I	Soluble	Serine protease cleaves C4b and C3b using C4bBP, CR1, Factor H, MCP or DAF as cofactors.	Classical, Alternative and Lectin
S Protein	Soluble	Binds soluble C5b67 and prevents insertion into cell membranes	MAC
Membrane inhibitor of reactive lysis (MIRL, CD59)	Membrane bound	Bind to C5b678 on autologous cells, blocking binding of C9	MAC

Table 1.7.1: Regulators of the complement system, their location, function and target. **Source:** ***Adapted from Goldsby et al., 2000***

DAF and CD59 are expressed on the plasma membrane of unfertilised human oocytes and preimplantation embryos while expression of MCP does not appear until the 4-to-8-cell stage, which is thought to coincide with genomic expression of the preimplantation human embryo (Fenichel *et al.,* 1995). Interestingly, all three of these complement regulators are expressed at the blastocyst stage of development, the stage at which ES cells are isolated (Fenichel *et al.,* 1995).

Following the demonstration that DAF, MCP and CD59 were highly expressed on foetal derived trophoblasts that come into direct contact with maternal blood and tissues, it has been suggested that regulators of complement play an important role in the protection of the human conceptus from maternal complement during pregnancy, (Holmes *et al.,* 1992; Holmes *et al.,* 1990). In rodents, a fourth complement regulator, complement receptor 1-related gene/protein y (*Crry*) is present and functions to regulate the deposition of C3 and C4 in a similar manner to MCP and DAF. In knockout studies, *Crry* deficiency was found to result in embryo lethality as a consequence of spontaneous activation of C3 and deposition of C3b on the trophoectoderm and ectoplacental cone (Xu *et al.,* 2000). While the majority of complement regulation on the developing foetus has been shown to be associated with expression of regulators by the placenta, arising from the trophoblast cells, expression of complement regulatory proteins has also been reported early in foetal development, in the developing liver for example (Simpson *et al.,* 1993) and thus may be expressed on human ES cells, which could affect the success of selective elimination strategies that utilise complement-mediated lysis.

1.7.2 Fluorescence Activated Cell Sorting (FACS)

The power of flow cytometry lies in its ability to analyse several parameters, on tens of thousands of individual cells within a few minutes, rather than relying on a bulk measurement from a whole population. The field of flow cytometry began in the 1930's and was gradually developed to become a commercially available technique by the mid 1970's (reviewed by Radcliff & Jaroszeski, 1998). Fluorescence activated cell sorting has been used to separate many different cell types (reviewed by Ormerod, 1999) and has recently been shown to successfully sort undifferentiated human ES cells (Eiges *et al.*, 2001).

The underlying principle of flow cytometry is that light is scattered and fluorescence is emitted, as light, from an excitation source, strikes a moving particle. All flow cytometers, whether for analysis or for cell sorting, function using four basic systems: fluidic, illumination, optical/electronic and data storage/analysis. The fluidic system is fundamental to flow cytometry and determines how light from the illumination system meets the moving particles. Briefly, sheath fluid, usually phosphate-buffered saline (PBS), is directed by air pressure through a flow chamber or nozzle, depending on the type of machine. The sample (as a single-cell suspension) is directed into the stream of sheath fluid and together they pass through the flow chamber/nozzle, as a sample stream. The pressure of the sheath fluid aligns the cells in single-file, a process referred to as hydrodynamic focusing, which consequently enables the light source to intersect individual cells. Illumination in the majority of flow cytometers comes via a laser beam, which has been directed to intersect the sample stream. Light scatter and fluorescent light emission occurs in all

directions (360°) when the focused laser beam strikes a moving particle within the stream. These signals are then quantified by the optical and electronic system.

Typically, flow cytometers can collect and quantify at least five different parameters. Two of these parameters are properties of light scatter; forward-angle light scatter (FSC) and side-angle (90°) light scatter (SSC). FSC is the result of diffraction, which provides basic morphological information such as relative size. SSC is the result of refraction and reflection and indicates granularity within the cytoplasm of cells, which can be indicative of cellular complexity. The remaining three parameters are associated with fluorescence emission. Most available cytometers allow examination of fluorescence from three different regions of the visible spectrum, Green (FL1) ~520nm, Orange-Red (FL2) ~570nm and Red (FL3) ~620nm. In this thesis the fluorochromes fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE) were used as probes, which are detected by the FL1 and FL2 channels respectively.

The final system, which is fundamental in the interpretation of flow cytometry experiments, is data analysis and presentation. The most common form of display for flow cytometry data is as a histogram. Histograms are used to display data from a single parameter and are very simple to interpret. Overlaying histograms can be used to effectively assess small differences in a single parameter between many different samples, as long as the data from the independent samples was acquired using the same parameter settings. In addition, data is often displayed either as a dot plot, which indicates individual events, or as a contour density plot, which shows

data for a population of cells using a series of concentric lines. The power of these two plots is that they allow the investigator to visualise two parameters (e.g. FSC and SSC or FL1 and FL2 fluorescence) simultaneously.

There can be a number of pit-falls with data generated by flow cytometry, if the experimental design was not carefully planned. The most important aspect of experimental design for flow cytometry is ensuring that the cytometer is appropriately configured. In order to obtain meaningful results, it is imperative that negative and positive experimental controls are used as reference points. Generally two negative control samples are required; one contains just the cell sample and is used to position the cells of interest, by adjusting the FSC and SSC, so that they appear on scale, while also setting a baseline for any cellular autofluorescence. The second negative control sample is used in situations where fluorochrome-conjugated antibodies are used as probes. Here the cell sample is treated either with a directly conjugated isotype control antibody, which has all of the properties of the monoclonal antibody but which is non-specific, or with the same secondary fluorochrome-conjugated antibody used for indirect staining but in the absence of the primary antibody. These negative controls can either be used to subtract non-specific fluorescence from the fluorescence values of experimental samples, or they can be used to determine a fluorescence threshold for judging positive/negative expression of the antigen of interest. In addition to negative controls, it is important that a positive control is also included; usually this takes the form of a cell line, which is known to stain strongly with the antibody of choice. Positive controls a) ensure the

success of labelling protocols and b) they provide an approximation of the fluorescence intensity that positively expressing experimental cells might have.

Analysis by flow cytometry using two or more fluorochromes has inherent complications that must be controlled for. Usually a fluorochrome will emit a spectrum of light that is strongest in a narrow bandwidth that corresponds to a defined detection channel. In addition weaker emission outwith the detection range will also be observed. It is this light emitted outside of the detection range of a particular channel that can cause complications when combinations of different fluorochromes are used for analysis. A consequence of overlapping spectral emission ranges is the detection of false-positives. This can be overcome by selecting fluorochromes that have minimal spectral overlap, or alternatively the cytometer can be adjusted electronically to compensate for spectral overlap. Compensation relies on controls, identical to the one being labelled with combinations of fluorochrome, which have been stained only with a single label. These controls are then analysed by the inappropriate channel, and the cytometer is adjusted to subtract any crossover fluorescence.

In addition to ensuring that the cytometer is appropriately configured there are also a number of other potential problems that investigators should be aware of. For example, investigators should be aware of the occurrence of false positives, which can result from the use of supra-optimal levels of fluorochrome-conjugated antibody. Antibodies should always be titrated on a positive control cell line and also on the experimental cell suspension, before they are used experimentally in flow cytometry.

Supra-optimal levels of antibody could result in the misinterpretation of experimental shifts in the mean peak values as a result of non-specific binding. In addition, when a sample suspension is very concentrated cells may exit the flow chamber/nozzle together and there may not be enough distance between them for the cytometer to resolve a single event at a time. This problem is referred to as coincidence, and can be avoided by either diluting the single cell suspension or by reducing the rate at which the sample passes through the cytometer.

When properly controlled, flow cytometry is a powerful tool for the collection of vast quantities of analytical data in a short space of time. In the case of rare events it is important that large numbers of events are processed at one time and that small changes are repeatedly observed before strong conclusions are drawn. However, cell sorting by flow cytometry could be a powerful alternative to complement-mediated lysis as a method for selectively removing minority populations of undifferentiated ES cells from a mixed population *in vitro*.

1.8 Project Objectives

A potential barrier to the clinical application of human ES-derived cells in regenerative medicine is the co-inoculation of undifferentiated human ES cells, with tumorigenic potential. The application of human ES-derived cell therapy therefore, requires the development of efficient strategies for the removal of undifferentiated ES cells from within a potentially therapeutic population. To address these concerns:

1) Engineered clones of undifferentiated human ES cells expressing defined cell-surface epitopes under the transcriptional regulation of hTERT or Oct4 promoters were isolated.

2) These clones were then used to investigate the use of complement-mediated lysis or cell sorting strategies for the elimination of contaminating undifferentiated ES cells from within a mixed population of human ES-derived cells. The efficiency of these strategies was verified by subsequent culture and analysis of recovered cells for the presence of undifferentiated ES cells.

CHAPTER 2 MATERIALS AND METHODS

MATERIALS

METHODS

2.1 Bacterial Transformation & Culture

2.2 Preparation of Plasmid DNA

2.3 DNA Manipulation and Subcloning

2.4 Maintenance of Cells in Culture

2.5 Estimation of Cell number

2.6 Storage of Cells

2.7 Manipulation of Cell Lines: Methods of Transfection

2.8 Characterisation of Normality and Pluripotentiality of Human ES cells

2.9 Analysis of Gene Expression

2.10 Strategies for the Selective Elimination of Human ES Cells

MATERIALS

All chemicals used were analar grade and purchased from BDH or Sigma unless otherwise stated.

Bacterial Transformation & Culture

Luria-Bertani (LB) Medium

1% Bacto-tryptone (Difco), 0.5% Bacto-yeast extract (Difco), 125mM Sodium chloride (NaCl).

LB-Agar

LB medium containing 1.5% agar (Difco).

SOC-medium

2% Bacto-tryptone, 0.5% Bacto-yeast extract, 10mM NaCl, 2.5mM potasssium chloride (KCl), 20mM Mg^{2+} stock (1M $MgCl_26H_2O$/ 1M $MgSO_47H_2O$) and 20mM glucose (Fisons).

Ampicillin Selection

Stock solution 50mg/ml used at concentrations of 50-100μg/ml

Kanamycin Selection

Stock solution 100mg/ml used at concentrations of 30-50μg/ml

Preparation of Plasmid DNA

Mini Prep Solutions (Promega)

Cell Resuspension Solution (Promega)

50mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 7.5, 10mM ethylenediaminetetraacetic acid (EDTA) and 100μg/ml RNase A

Cell Lysis Solution (Promega)

200mM sodium hydroxide (NaOH), 1% (w/v) sodium dodecyl sulphate (SDS)

Neutralising Solution (Promega)

1.32M potassium acetate pH 4.8

Column Wash Solution (Promega)

80mM potassium acetate, 8.3mM Tris-HCl pH 7.5 40μm EDTA, 55% absolute ethanol

Maxi Prep Solutions (Qiagen)

Re-suspension buffer P1 (Qiagen)

50mM Tris-HCl pH 8.0, 10mM EDTA and 100μg/ml RNase A

Cell lysis buffer P2 (Qiagen)

200mM NaOH, 1% SDS (w/v)

Neutralisation buffer P3 (Qiagen)

3M Potassium acetate pH 5.5

Equilibration buffer QBT (Qiagen)

750mM NaCl, 50mM 3-[N-morpholino]propanesulfonic acid (MOPS) pH 7.0, 15% isopropanol (v/v) and 0.15% Triton X-100 (v/v)

Column Wash buffer QC (Qiagen)

1.0M NaCl, 50mM MOPS pH 7.0 and 15% isopropanol (v/v)

Elution buffer QF (Qiagen)

1.25M NaCl, 50mM Tris-HCl pH 8.5 and 15% isopropanol (v/v)

TE Buffer

10mM Tris-HCl pH 8.0 and 1mM EDTA.

DNA manipulation and Subcloning

Restriction Digest

Buffer H (Roche)

500mM Tris-HCl, 1M NaCl, 100mM magnesium chloride ($MgCl_2$), 10mM Dithioerythritol, pH 7.5 at 37°C

SEBuffer B (SibEnzyme)

10mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol (DTT) pH 7.6 at 25°C

Agarose Gel Electrophoresis

10X TBE Buffer

0.89M Tris-HCl pH8.0, 0.89M borate and 0.02M EDTA

Type III DNA Loading dye (6X)

0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol

Gel Purification (QIAquick)

Elution Buffer (Qiagen)

10mM Tris-HCl, pH 8.5

Cell Culture

Maintenance of Cell Lines

Sterile Phosphate Buffered Saline [PBS]

0.16M NaCl, 0.003M KCl, 0.008M disodium hydrogen phosphate and 0.001M potassium dihydrogen phosphate in distilled water, supplied in tablet form (Oxoid). Reconstituted in distilled water (dH_2O) and autoclaved prior to use.

Trypsin/EGTA (TEG) Solution.

92.7mM NaCl, 0.845mM di-sodium hydrogen orthophosphate (Na_2HPO_4), 1.58mM potassium dihydrogen orthophosphate (KH_2PO_4), 4.46mM KCl, 5mM D-glucose (Fisons), 22.28mM Tris-HCL, 0.0009% Phenol red, 0.25% trypsin, 1.05mM ethylenebis(oxyethylenenitrilo)tetraaceetic acid (EGTA) and 0.000105% polyvinyl alcohol, in distilled water. Adjust pH to 7.6.

EDTA/PBS

0.5mM EDTA in sterile PBS and filter sterilised.

Collagenase IV

234U/mg collagenase IV (Gibco) in KO-DMEM (1mg/ml) filter sterilised.

Trypsin/EDTA (TED) Solution (Sigma)

0.5g/l porcine trypsin and 0.2g/l EDTA in Hanks' Balanced Salt Solution with phenol red

Basic Fibroblast medium

Dulbecco's modified eagles medium (DMEM; Sigma) supplemented with 10% (v/v) foetal bovine serum (FBS; Globepharm), 0.1mM Non-Essential Amino Acids (NEAA; Gibco) and 2mM L-glutamine (Gibco).

Basic human ES medium

Knock-Out (KO) DMEM (Gibco) supplemented with 20% (v/v) KO-serum replacement (Gibco), 0.1mM NEAA, 2mM L-glutamine and 0.1mM β-mercaptoethanol (Gibco). Recombinant human basic fibroblast growth factor (hbFGF; Sigma) was added fresh at 4ng/ml.

Quench medium

KO-DMEM supplemented with 10% (v/v) FBS.

PDFF medium

Glasgow's minimum essential medium (GMEM; Sigma) supplemented with 10% (v/v) FBS, 2mM L-glutamine and 0.1mM NEAA

RPMI- complete medium

Roswell Park Memorial Institute Medium 1640 (RPMI-1640; Sigma) supplemented with 10% (v/v) FBS and 2mM L-glutamine

Complete 199 Medium

Medium-199 (Sigma) supplemented with 10% (v/v) FBS, 0.1mM NEAA and 2mM L-glutamine

1x Penicillin/Streptomycin

100U/ml penicillin and 100μg/ml streptomycin, purchased in solution from Gibco.

2x Freeze mix

Specific for each cell line, but in general 50% (v/v) complete culture medium supplemented with 30% (v/v) serum and 20% dimethyl sulfoxide (DMSO) (Table 2.6.1).

G418 (geneticin)

Stock solution made by dissolving G418 Sulphate (PAA Lab, GmbH Austria) in distilled water to 100mg/ml. Filter sterilised through a 0.2μm syringe filter and stored at –20°C.

In Vitro Differentiation

Basic Differentiation Medium

KO-DMEM supplemented with 10% (v/v) FBS, 0.1mM NEAA, 2mM L-glutamine and 0.1mM β-mercaptoethanol.

Osteogenic Differentiation Medium

Basic differentiation medium supplemented with 10mM β-glycerophosphate 50μM ascorbic acid and 0.1μM dexamethasone.

Cytogenetic Analysis

Hypotonic Solution

0.56% (w/v) KCl in distilled water.

2X SSC

0.3M Sodium chloride and 0.03M tri-sodium citrate dissolved in distilled water.

Giemsa stain

Diluted to 10% in Gurrs R66 phosphate buffer pH 6.8, made with Gurrs buffer tablets (Gibco).

Flow Cytometry

FACS Blocking Buffer

40% (v/v) heat inactivated rabbit serum in FACS staining buffer

FACS Staining Buffer

PBS supplemented with 2% (v/v) FBS and 2mM EDTA

Immunochemistry

Paraformaldehyde

10% (w/v) paraformaldehyde (PFA) was dissolved in heated PBS as a stock solution. Dilutions to 4%, 1% and 0.1% were made in PBS for experimental use.

With Antibody

Blocking serum

10% (v/v) normal serum (as appropriate) in PBS supplemented with 0.1% polyoxyethylene sorbitan monolaurate (Tween-20). Blocking serum was diluted 1:10 (1%) for antibody dilutions

With Lectin

Wash Solution

Complete sterile PBS (containing Mg^{2+}/Ca^{2+}) supplemented with 1% (v/v) bovine serum albumin (BSA; Gibco).

BS-IB$_4$ Stain

200µg of fluorescein isothiocyanate conjugated *Bandeiraea simplicifolia* isolectin B4 (*BS-IB$_4$*; Sigma) dissolved in complete PBS supplemented with 10% (v/v) newborn calf serum (NCS) to a final concentration of 5µg/ml. For flow cytometry PBS minus Mg^{2+}/Ca^{2+} was used.

With Giemsa

Giemsa (BDH) was diluted to 10% in water.

With Acridine Orange and Ethidium Bromide (AO:EtBr)

AO:EtBr solution

10mg/ml stock solutions of acridine orange and ethidium bromide (Sigma) diluted in complete PBS to 0.2mg/ml

Protein Analysis

Protein Lysis Buffer

50mM Tris-HCl pH7.6, 140mM NaCl and 1% (v/v) Triton-X 100, in sterile distilled water. Immediately prior to use, one protease inhibitor cocktail tablet (Roche) was dissolved in 10mls of lysis buffer.

2X SDS loading buffer

100mM Tris-HCL pH6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol in distilled water. 200mM dithiothreitol (DTT; Biorad) was added as appropriate.

10X Tris-Glycine Running Buffer

250mM Tris-HCl, 2.5M glycine and 1% SDS pH adjusted to 8.3 with concentrated hydrochloric acid (HCl) in distilled water.

Western Transfer Buffer

0.5M Tris-HCl, 3.84M glycine pH adjusted to 8.3 with concentrated HCl in distilled water. 20% methanol added to 1X buffer prior to use.

Immunoblotting Blocking solution

5% (w/v) skimmed milk powder (Marvel) with 1% (v/v) FBS, 1% (v/v) BSA and 0.1% (v/v) Tween-20 in PBS.

Immunoblotting Wash Solution

0.1% Tween-20 in PBS

METHODS

2.1 Bacterial Transformation & Culture

2.1.1 Bacterial Strains

In the course of this thesis a variety of bacterial strains were used to perform specific functions. Subcloning efficiency DH5α competent cells (Invitrogen) and XL1-Blue competent or subcloning grade competent cells (Stratagene) were used interchangeably for routine circular plasmid transformation and following simple cloning steps such as small or sticky ended insert ligations. However, following complex cloning steps of large fragments or blunt ended inserts, XL10-Gold ultracompetent cells (Stratagene) were used.

2.1.2 Transformation of Competent Bacteria

The following is an example of a transformation protocol, however, there are discrete differences in the protocol for the different bacterial strains and these can be found in Table 2.1.1.

Ultracompetent cells (*Epicurian Coli,* XL10-Gold, Strategene) were thawed slowly on ice. The cells were dispensed into pre-cooled 1.5ml microcentrifuge tubes containing 100µl volumes to which 4µl of β-mercaptoethanol (Strategene) was added. The cells were incubated on ice for 10 minutes, with gentle mixing every 2 minutes. 2µl of a ligation reaction or plasmid DNA was added to one tube and as a control 2µl of pUC18 DNA was added to a second tube. Tubes were then incubated on ice for 30 minutes before being exposed to a 30 second heat shock at 42°C. The tubes were then returned to ice for a further 2 minutes before adding 900µl of pre-

heated (42°C) SOC medium. Tubes were then incubated at 37°C for 1hour with gentle agitation at 180rpm, before being plated (100µl) on selective Luria-Bertani (LB) agar.

	Volume of cells required	Addition of β-mercaptoethanol	Incubation with DNA on ice	Heat shock	Temperature of Heat Shock	Period of Incubation at 37°C
DH5 α	50µl	None	20 min	20 sec	37°C	1hr
XL1-Blue competent	100µl	1.7µl (supplied)	30 min	45 sec	42°C	1hr
XL1-Blue Subcloning grade	50µl	None	20 min	45 sec	42°C	30 min
XL10-Gold	100µl	4µl (supplied)	30 min	30 sec	42°C	1 hr

Table 2.1.1: Specific bacterial strain differences in transformation protocol.

2.2 Preparation of Plasmid DNA

2.2.1 Small Scale (Mini) Preparation

Small scale DNA isolation was performed using the Wizard *Plus* Minipreps System (Promega). Plasmid DNA was extracted from individually picked bacterial colonies incubated for 12-16 hours in a 15ml polypropylene tube containing 3-5ml of LB medium supplemented with an appropriate antibiotic at 37°C with gentle agitation at 180rpm.

Cultures were harvested by centrifugation (10,000g; 10minutes) and the supernatant discarded. Bacterial pellets were re-suspended in 300µl of resuspension solution and transferred to 1.5ml microcentrifuge tubes prior to the addition of an equal volume of cell lysis solution. The suspension was mixed gently by inversion and incubated at room temperature for 5 minutes. The addition of neutralising solution (300µl)

stopped the lysis reaction before centrifugation (10,000g; 5 minutes; room temperature). The supernatant (clear lysate) was passed through a resin solution in a mini-column/syringe assembly, by vacuum, and the flow-through discarded. The columns were then washed with 2ml of column wash solution, again by vacuum and the flow-through discarded. The columns were transferred to 1.5ml microcentrifuge tubes and excess column wash removed by centrifugation (10,000g; 1 minute; room temperature). DNA was eluted by the addition of 100µl of sterile dH_2O and centrifugation (10,000g; 1 minute; room temperature).

2.2.2 Large Scale (Maxi) Preparation

Large scale DNA isolation was performed using the Qiagen Plasmid Maxi Kit (Qiagen) this procedure is designed to yield 500µg of DNA. A single bacterial colony, grown on selective LB agar plates, was picked and used to inoculate a 3ml starter culture of selective LB medium in a 15ml polypropylene tube. This culture was incubated at 37°C with gentle agitation (180rpm) for 8 hours before being used to inoculate 100ml selective LB medium, which was grown for 12-16 hours (with agitation (180rpm) at 37°C).

Cultures were harvested by centrifugation (6000g; 15 minutes; 4°C) and the supernatant discarded. The bacterial pellet was resuspended in 10ml of chilled (4°C) resuspension buffer P1. Following resuspension, 10ml of cell lysis buffer P2 was added and mixed by gentle inversion and incubated at room temperature for 5 minutes. Chilled (4°C) neutralisation buffer P3 (10ml) was added mixed thoroughly by gentle inversion and incubated on ice for 15 minutes. Cell lysates were collected

by centrifugation (20000g; 30 minutes; 4°C) and the supernatants filtered through 3MM paper (Whatman). A QIAGEN-tip 500 was equilibrated with 10ml of buffer QBT and the filtered supernatant was allowed to pass through the QIAGEN-tip column by gravity. The QIAGEN-tip was then washed twice with 30ml buffer QC and the DNA eluted with 15ml of buffer QF into a cortex tube. DNA was precipitated by gently mixing 0.7 volumes of isopropanol (room temperature) to the elution followed by centrifugation at 15000g for 30 minutes at 4°C. The pellet was washed with 1ml 70% ethanol (room temperature), collected and transferred to a 1.5ml microcentrifuge tube, before being centrifuged at 10000g for 5 minutes. The 70% ethanol wash was repeated and once removed the pellet was air-dried before being resuspended in 0.5-1ml of TE buffer.

2.2.3 DNA Quantification

Spectrophotometric measurements were used to determine the quantity and quality of the DNA preparations. Typically a DNA sample was diluted 1:50 with TE buffer and the absorbance read at 260nm to give the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50μg/ml of double stranded DNA. The ratio between readings at 260nm and 280nm provided an estimation of the purity and hence quality of the DNA preparation. Pure DNA has an OD_{260}/OD_{280} ratio of 1.8; contamination with protein produces a lower ratio.

2.3 DNA Manipulation and Subcloning

2.3.1 Restriction Endonuclease Digestion

Plasmid DNA was digested with 5 units of restriction enzyme for every μg of DNA in a microcentrifuge tube using the supplied manufacturer's buffer and instructions. Typically the final reaction volume was 50μl. Tubes were incubated at the appropriate temperature, usually 37°C, for 2 hours or until a complete digestion was observed by agarose gel electrophoresis. Restriction endonucleases were obtained from either Roche or New England BioLabs Inc (NEB).

2.3.2 Agarose Gel Electrophoresis

Agarose powder was dissolved in 1X TBE (0.8-1.2% w/v) by microwave heating and allowed to cool. Ethidium bromide (0.5μg/ml) was added to the agarose prior to pouring the gel using horizontal, buffer-immersed electrophoresis gel kit (Anachem). DNA samples were mixed with 6X Type III loading dye before loading into the lane wells; at least one lane was reserved for 5μl of molecular weight marker (Hyperladder Type I, Roche). An electrical current between 30 and 100volts was applied. Following electrophoresis, DNA was visualised using a UV transilluminator and images captured using Multi-Analyst software (BioRad).

2.3.3 Purification of DNA fragments from Agarose Gels

DNA fragments were electrophoresed through an agarose gel, excised using a clean sharp scalpel (visualised under long-wavelength UV light) and transferred to a microcentrifuge tube. The gel slice was then processed using a QIAquick column (Qiagen) in accordance with the manufacturer's instructions.

The weight of the agarose was determined and 3 volumes of buffer QG to 1 volume of gel were added (e.g. 300µl buffer to 100mg gel). This was incubated at 50°C for approximately 10 minutes until the gel slice had dissolved. To the mix, 1 gel volume of isopropanol was added and the sample transferred to a QIAquick column held within a 2ml collection tube. The QIAquick column was then microcentrifuged at 10,000g for one minute to bind the DNA. This step was repeated a second time to increase DNA yield, after which the flow-through was discarded. The column was washed with 750µl of buffer PE for 5 minutes to remove any remaining agarose and excess salts, followed by a third 1 minute microcentrifugation. The flow through was discarded and the column re-centrifuged to remove any remaining buffer (1 minute). The column was then placed in a clean 1.5ml microcentrifuge tube. 30µl of elution buffer was added and the column was allowed to stand for 1 minute before being microcentrifuged for a final 1 minute to elute the DNA.

2.3.4 Cloning by Polymerase Chain Reaction (PCR)

High-fidelity PCR using *PfuTurbo* DNA Polymerase (Stratagene) was used to clone the H-2K^k cDNA using pMAC KK II (Milteny Biotech) plasmid DNA as a template. During preparation of the reaction all reagents (Table 2.3.1) were maintained on ice and the final reaction mix overlaid with mineral oil, to prevent evaporation. PCR reactions were placed in a thermocycler (Hybaid MBS 0.2) and cycled through the temperatures specified in Table 2.3.1.

Primers	50µl Reaction Mix		PCR Machine/Program
Fwd Primer: GATGGCACCCTGCATGCTGCT **Rev Primer**: GGATCTACCCTCCTTTTCCACCTGTGTTTC	Template DNA	10ng	*Hybaid MBS 0.2*
	PCR Buffer	10X	
	F. Primer	100pM	60s at 95°C
	R.Primer	100pM	30s at 95°C } 20 cycles
	$MgCl_2$	1mM	60s at 63°C } 20 cycles
	Mixed dNTPs	200µM	60s at 72°C } 20 cycles
	PfuTurbo	25Units	180s at 72°C
	Sterile dH_2O	to 50µl	

Table 2.3.1: PCR Conditions for Cloning the H2-K^k cDNA from the pMAC Kk II Plasmid (Milteny Biotech). Primers were produced by MWG.

PCR with *PfuTurbo* produces a blunt-end DNA fragment, thus the H2-K^k cDNA was treated with Taq DNA polymerase (Roche), which adds a single deoxyadenosine to the 3'-ends of the amplified fragment. The resulting "A-tail" allows very efficient cloning of the PCR product into the TA vector system (Promega). Briefly, the PCR fragment was precipitated (2.3.5.2) from the reaction mix and resuspended in 7µl of sterile dH_2O. Added to this was 10X Roche Taq buffer plus magnesium chloride ($MgCl_2$), 2mM mixed dNTP and 5 units of Taq (Roche) in a final volume of 10µl. The reaction was incubated at 70°C for 15 minutes before running and purifying the fragment by agarose gel electrophoresis and gel purification (section 2.3.3). The purified DNA fragment was then ligated (section 2.3.5) into the TA cloning system (pGEM-TEasy Promega). The advantage of the pGEM-TEasy vector system was the multiple cloning sites at each end of the integration site, thus providing multiple options for subsequent cloning steps.

2.3.5 Ligation

DNA ligations were performed using the Rapid DNA Ligation Kit (Roche). This system enables ligation of both sticky- and blunt-ended DNA fragments in less than 10 minutes at room temperature. Ligation reactions contained no more than 250ng of total DNA (vector + insert) in a total reaction volume of 21µl. The molar ratio of vector to insert was typically 1:5. However, ratios of 1:3 and 1:20 were occasionally employed, depending on the size of the DNA insert fragment.

The following example describes a typical ligation reaction using a 1:5 vector to insert ratio. 1ng vector DNA (10kb) and 50ng of DNA insert (1kb) were dissolved with 2µl of 5x DNA Dilution Buffer (Roche) in a total volume of 10µl (sterile dH_2O was added as required). After gentle mixing, 10µl of 2x T4 DNA Rapid Ligation Buffer (Roche) was added and mixed thoroughly. Finally the reaction was supplemented with 5 units (1µl) of T4 DNA Ligase (Roche), mixed gently, and incubated at room temperature for 5minutes, before being transformed into competent bacteria (section 2.1.2).

2.3.5.1 Vector Backbone De-phosphorylation

Prior to ligation, vector backbones with compatible-ends were incubated (1hour at 37°C) with shrimp alkaline phosphatase (SAP) (Roche) in order to de-phosphorylate them, preventing self-ligation. SAP was removed by ethanol precipitation of DNA (section 2.3.5.2).

2.3.5.2 Ethanol Precipitation

DNA samples were precipitated with 0.1 volume of 3M Sodium Acetate pH5.5 and 2.5 volumes of absolute ethanol, mixed and then chilled at –20°C for 1-2 hours. The DNA was pelleted by microcentrifugation (10,000g, 20 minutes at room temperature) and the supernatant discarded. The DNA pellet was then washed twice with 70% v/v ethanol (centrifuged at 10,000g for 2 minutes between washes) to remove salts. The pellet was then air-dried and resuspended in an appropriate buffer (TE or sterile dH_2O). Care was taken to dry the pellet sufficiently, as ethanol inhibits certain enzymatic reactions, but not to over-dry the pellet as this can affect the ability of DNA to go into solution. DNA samples were stored at –20°C.

2.3.5.3 Generating Compatible Ends

Where required, blunt ends from 3' overhangs were generated using Klenow (Roche). This was achieved by incubating the DNA fragment with 1-5 units of Klenow enzyme in its own reaction buffer and 10mM of mixed dNTP's in a total reaction volume of 50µl for 20 minutes at room temperature. The reaction was stopped by removal of the enzyme through ethanol precipitation (section 2.3.5.2).

2.4 Maintenance of Cells in Culture

2.4.1 Human Embryonic Stem Cells (hES)

Human embryonic stem cells were cultured in a feeder-free culture system, with media "conditioned" by a mouse embryonic feeder layer using a protocol adapted from that of Xu et al., (2001). Murine embryonic fibroblasts (MEFs) were derived from mid-gestation foetuses (13.5 days) and maintained in culture to produce "conditioned" media (CM) for the maintenance of human ES cells. An F1 hybrid strain (C57Bl/6 x CBA) was used extensively and was found to produce the best quality CM in our hands.

2.4.1.1 Derivation of Mouse Embryonic Fibroblasts (MEFs)

The uterine horns of a pregnant female mouse were taken at day 13.5 of pregnancy and the foetuses removed into sterile PBS, supplemented with 200U/ml penicillin/200µg/ml streptomycin. Each individual foetus was dissected away from the placenta, decapitated and had the soft red viscera removed. The remaining soft body tissue was then subjected to a cycle of enzymatic digestions and physical disaggregation. Individually the foetuses were incubated with 2ml of Trypsin/EGTA (TEG) for 5 minutes at 37ºC and briefly vortexed to help disaggregate the cells, before being returned to the incubator for a further 5 minutes. This cycle was repeated 2-3 times, until the majority of the body tissue had been disaggregated. To this cell suspension 3ml of basic fibroblast medium (DMEM supplemented with 10% (v/v) Foetal Bovine Serum (FBS), 2mM L-glutamine and 1% (v/v) Non Essential Amino Acids (NEAA)) was added, to neutralise the actions of trypsin, and the cells vortexed for a final time. Large aggregated clumps of cellular debris were allowed to

settle before the supernatant (single cell suspension) was collected and transferred to a plastic cell culture treated flask (NUNC). Typically, the cell suspension produced by 1 foetus was used to seed a single 80cm^2 flask.

MEFs were maintained in culture (section 2.4.1.2) for at least 1 passage after derivation, in the absence of antibiotics, prior to freezing (section 2.6), to reduce the risk of passing microbial contamination to other cell lines in culture.

2.4.1.2 Maintenance of Mouse Embryonic Fibroblasts

MEFs were cultured in basic fibroblast medium directly on tissue culture plastic (NUNC) at 37°C in a humidified incubator with 5% CO_2. Expansion of MEFs was necessary and passage was performed when cells reached 90% confluence as follows. Culture media was aspirated and cells washed with PBS to remove traces of serum, if left serum reduces the efficiency of the enzymatic digestion. The cells were then incubated with a minimal volume of TEG solution for 2-3 minutes, or until the cells had rounded and become refractive. The cells were further encouraged to detach by gently tapping the side of the flask. Immediately after detachment, serum-containing medium was added to neutralise the effects of the trypsin. The cells were collected by centrifugation at 200g for 5 minutes, after which the supernatant was discarded and the cell pellet resuspended in basic fibroblast medium before being transferred to a new tissue culture flask (NUNC) of appropriate volume. Typically MEFs would grow in culture for 4 passages before they senesced.

2.4.1.3 Production of Conditioned Media (CM) from Mouse Embryonic Fibroblasts

It is hypothesised that feeder-cells produce/secrete a growth factor when they are exposed to basic human ES medium (KO-DMEM (Gibco) supplemented with 20% (v/v) KO serum replacement (Gibco), 1% NEAA (Gibco), 1mM L-glutamine (Gibco) and 0.1mM β-mercaptoethanol (Gibco)), in response to the presence of recombinant human basic fibroblast growth factor (hbFGF), and that this currently elusive factor is essential for the maintenance of pluripotent human ES cells.

This media, referred to as conditioned medium (CM), was made by removing MEF cultures at approximately 80% confluence, from their normal culture medium and replacing it (after a PBS wash) with basic human ES media containing 4ng/ml hbFGF (Sigma). After 18-24 hours of exposure to the MEFs, the CM was retrieved, filtered through a 0.2μm low protein-binding filter (Nalgene) and supplemented with addition L-glutamine (1mM) and a further 4ng/ml hbFGF before being used to culture human ES cells. Excess CM was stored at -20°C and used at later dates, media that had been stored for more than 2 months was discarded.

2.4.1.4 Culture Conditions for Human Embryonic Stem Cells

In order to maintain human ES cells in an undifferentiated state they were cultured on growth factor reduced (GFR) matrigel (Becton Dickinson), a matrix of laminin, collagen IV, entactin and heparan sulphate proteoglycan. Matrigel was thawed on ice and diluted 1:100 in cold KO-DMEM (Gibco) before being used to coat tissue culture treated plastic vessels (NUNC). Polymerisation of the matrigel took 1 hour at room temperature, after which the solution was removed and the plastic ware washed

to remove any excess. Human ES cells were then seeded at the desired density and grown at 37°C in a humidified incubator with 5% CO_2 and fed daily with conditioned medium.

The expansion of human ES cells was performed in a similar manner to that of MEFs (section 2.4.1.2) using TEG, however, in the course of this study it has been necessary to use different passaging regimes including the use of collagenase IV treatment and EDTA treatment, the differences between which are outlined in Table 2.4.1.

Typically, human ES cells were passaged when they reached 70-95% confluence, depending on the passaging regime. The culture medium was removed and when appropriate the cells were washed with KO DMEM to remove dead cells and debris. Cells were then incubated with the appropriate disaggregation agent at 37°C and monitored periodically under the microscope. The duration of the incubation with the disaggregation agent was governed by the cells; when they started to round up and become refractive the agent was disabled as appropriate (Table 2.4.1). Following centrifugation the human ES cells were resuspended in CM and plated at the required density on matrigel coated tissue culture plastic and cultured at 37°C in a humidified incubator with 5% CO_2. Culture medium was replenished daily.

Disaggregation Agent	Level of Confluence	Disabled By:	Method of cell removal	Comments
Collagenase IV	At least 70%	Removed	Scraping (Greiner Bio-one)	Over-exposure to collagenase results in the loss of colony cells. Remaining cell clumps become too small and result in a low plating efficiency.
EDTA/PBS (EDTA)	80-90%	Quenched	Both Tapping and scraping	If cells are allowed to become too confluent EDTA disaggregation is less efficient, cells become clumpy and don't seed well.
Trypsin/EGTA (TEG)	85-95%	Quenched	Tapping followed by centrifugation	Over-exposure to TEG results in increased cell lysis and the formation of clumps. If not exposed for long enough, cells refuse to detach from their matrix and have to be mechanically removed

Table 2.4.1: Variation between different passaging regimes used for the culture of undifferentiated human ES cells.

2.4.2 Human Multipotential Erythroid Progenitor Cells (K562) and Human Promonocytic Cells (U937)

Both cell lines were obtained frozen from the European Collection of Cell Culture (ECACC, Salisbury, Wiltshire, UK) and tested for mycoplasma. Cells were cultured in accordance with the protocol supplied. Briefly, cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS and 2mM glutamine (referred to as RPMI complete medium) in suspension at a cell density of between 1-10x10^5 cells/ml (K562) and between 2-9x10^6 cells/ml (U937). Suspension cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and were passaged every 2-3 days by adding fresh medium and returning the cells to their proliferative cell density. The cells were harvested by centrifugation at 200g for 5 minutes.

2.4.3 Human Embryonic Kidney Cells (HEK 293)

HEK 293 cells were obtained from Dr Wei Cui (Roslin Institute, Edinburgh), and maintained in DMEM supplemented with 10% (v/v) FBS, 2mM L-glutamine and 1% (v/v) NEAA. Monolayer cultures were maintained directly on tissue culture plastic (NUNC) at 37°C in a humidified incubator with 5% CO_2, medium was refreshed every 48 hours. Cells were passaged when they reached 80-90% confluence as described for MEF (see section 2.4.1.2) except that Trypsin/EDTA (TED) was used for disaggregation.

2.4.4 Ovine Foetal Fibroblasts (PDFF)

Ovine foetal fibroblasts (FF) from the Polled Dorset (PD) breed were derived by Dr Angelika Schnieke (PPL Therapeutics, Roslin, Midlothian). Monolayer cultures of PDFF were maintained in GMEM supplemented with 10% (v/v) FBS, 2mM L-glutamine and 1% NEAA, on 0.1% gelatin-coated tissue culture plastic (NUNC) at 37°C in a humidified chamber with 5% CO_2. Medium was replenished every 48 hours and cells were passaged using TEG as described for MEF (see section 2.4.1.2) when they reached 80-90% confluence.

2.4.5 Porcine Kidney Epithelial Cells (LLC-PK1)

LLC-PK1 cells were obtained from the ECACC and tested for mycoplasma. Monolayer cultures were maintained directly on tissue culture plastic (NUNC) at 37°C in a humidified incubator with 5% CO_2, in Medium 199 (Sigma) supplemented with 10% (v/v) FBS, 2mM L-glutamine and 1% (v/v) NEAA that was refreshed every 48 hours. Cells were passaged when they reached 80-90% confluence as

described for MEF (2.4.1.2) except that Trypsin/EDTA (TED, Sigma) was used as a disaggregation agent.

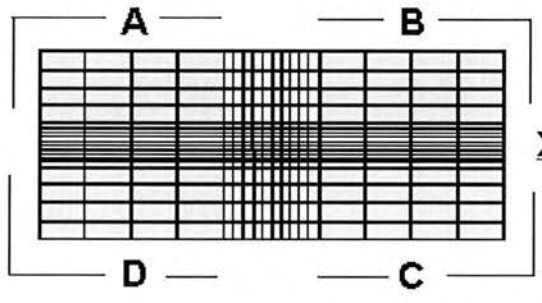
2.4.6 Derivation and Maintenance of Adult Murine Fibroblasts

It was necessary to isolate fibroblasts from adult tissues of the CBA and Balb C mouse strains, to act as positive and negative controls, respectively, for H2-K^k expression. Lung, liver and spleen tissues were used for this isolation.

All tissues were collected and washed 3 times in sterile PBS containing 200U/ml penicillin/200μg/ml streptomycin to reduce the risk of bacterial contamination. The tissues, maintained in 1-2ml of sterile PBS, were then macerated into small pieces using mincing scissors before being passed through a size 18G needle, in an attempt to titurate the tissues and free the cells. The macerated tissue was then re-suspended in 4ml of TEG and incubated at 37°C for 3-5 minutes. The trypsin was inactivated by the addition of 5-10ml of serum containing medium, which was then mixed by vortex for 1 minute before being centrifuged at 200g for 5 minutes. The supernatant was discarded and the pellet resuspended in 10-20mls of basic fibroblast media, depending on the amount of tissue collected. Persistent large clumps of tissue were allowed to settle for 5 minutes and the remaining suspension plated on to tissue culture plastic (NUNC) to adhere. Culture media was replaced 48hours after isolation and the cells were subsequently maintained in the same manner as MEFs (see section 2.4.1.2).

2.5 Estimation of Cell number

Cells were harvested as previously described (section 2.4) and generally resuspended in 5-10ml of appropriate medium. After mixing the suspension thoroughly, a sample was counted using a haemocytometer. A total of 64 squares containing cells were counted, averaged and the cell number determined using the formula in Figure 2.5.1



$$\frac{\Sigma\left((A1\text{-}16) + (B1\text{-}16) + (C1\text{-}16) + (D1\text{-}16)\right) \times 10^4}{4} = \underline{\text{Cells/ml}}$$

Figure 2.5.1: Formula for counting cells using a haemocytometer

Cell viability was determined using trypan blue staining. A 1:1 mix of 0.5% trypan blue and cell suspension was made immediately prior to counting. Viable cells exclude the blue dye while non-viable cells take it up. The percentage of viable cells was then determined.

2.6 Storage of Cells

Subconfluent monolayer cultures or log-phase growth suspension cultures were harvested and pelleted as previously described (2.4). The cell pellets were then resuspended in complete culture media to which an equal volume of 2x freezing mix was added, for difference between cell lines see Table 2.6.1. One ml aliquots were

transferred to 2ml cryotubes (Sarstedt) and frozen overnight at –80°C before being transferred to –150°C freezers for long-term storage.

Cell Line	Complete medium component	Serum/Serum replacement component	DMSO component
Mouse Embryonic fibroblasts (MEF)	50% basic fibroblast medium	30% FBS	20%
Human Embryonic Stem Cells (hES)	50% CM	30% KO Serum Replacement	20%
Human Multipotential Erythroid Progenitor Cells (K562)	No medium, pellet resuspended in 2x freeze mix alone	90% FBS	10%
Human Promonocytic Cells (U937)	No medium, pellet resuspended in 2x freeze mix alone	90% FBS	10%
Porcine Kidney Epithelial Cells (LLC-PK1)	No medium, pellet resuspended in 2x freeze mix alone	90% FBS	10%
Human Embryonic Kidney Cells (HEK 293)	50% basic fibroblast medium	30% FBS	20%
Ovain Foetal Fibroblasts (PDFF)	50% PDFF complete medium	30% FBS	20%

Table 2.6.1 Components of the 2x freezing mix for the different cell lines used in this thesis. Following the addition of the freeze mix, all cells were stored at -80°C for 24 hours before being transferred to -150°C freezers for long-term storage.

Cells recovered from long-term storage (-150°C) were thawed rapidly at 37°C, and resuspended in appropriate basic culture medium. The cells were then pelleted at 200g for 5 minutes to remove traces of DMSO and plated out in complete growth medium as previously described (section 2.4). With the exception of non-adherent cells, the medium was always refreshed 16-24 hours after resuscitation.

2.7 Manipulation of Cell Lines: Methods of Transfection

2.7.1 Lipofection

In the early stages of this thesis lipofection was the only option for the genetic manipulation of human ES cells. The lipofection protocols were robust and provided good transfection frequency for both transient and stable modifications. However, lipofection favours multi-copy integration, which increased the risk of gene silencing through the recognition of repeat sequences (as discussed in section 1.3.5.3).

Human ES cells were transfected using the Lipofectamine 2000 (LF2000) system (Invitrogen) following a modified manufacturer's protocol provided by Geron Corporation. Human ES cells were seeded at a density of $1x10^5$ cells per well of a 6-well plate and cultured for 48 hours (to give 90% confluence) prior to the introduction of DNA.

For each well, 5μg of either circular (transient transfection) or linear (stable transfection) DNA was diluted in OPTI-MEM (Invitrogen) to give a final volume of 250μl. Similarly, 12μl of LF2000 reagent was also diluted in OPTI-MEM to a final volume of 250μl. The diluted DNA and LF2000 were then combined (500μl total volume) and incubated for 30 minutes at room temperature to allow DNA-LF2000 reagent complexes to form. The culture media from the proliferating human ES cells was then replaced with 2.5ml of normal CM+hbFGF plus 500μl of DNA-LF2000 reagent complexes. The cells were incubated with these complexes for not more than 16 hours at 37°C in a humidified incubator with 5% CO_2.

2.7.2 Electroporation

The conditions for a successful electroporation vary between cell types. Table 2.7.1 provides details of the specific conditions for each of the cell lines used in this thesis. Monolayer and suspension cultures were harvested as described in section 2.4, counted and resuspended at the required cell density in 750µl of the appropriate buffer (see Table 2.7.1).

Cell Line	Quantity of Cell	Quantity of DNA	Volts	µF/µS	Buffer	Reference
H7 & H9	$1x10^6$	50µg	300	100µS	Hyposmolarity buffer (eppendorf)	
K562	$1x10^7$	20µg	250	960µF	PBS	Nakano *et al*, 2003
U937	$1x10^7$	20µg	250	960µF	PBS	Based on protocol for K562
HEK 293	$8x10^5$	25µg	225	750µF	Electroporation buffer (50mM K_2HPO_4, 20mM KAc, 25mM $MgSO_4$, pH 7.35)	Adelsberger *et al.*, 2000

Table 2.7.1 Cell Line Specific Electroporation Conditions. Electroporation of human ES cells used the Eppendorf multiporator with conditions defined within the McWhir Group. All other cell types were electroporated using the BioRad Pulse electroporator.

Human ES cells were electroporated using the Eppendorf multiporation system, which involved incubating the cells in hyposmolarity buffer at room temperature for exactly 20mins to swell the cells prior to delivery of the DNA. 50µg of DNA (circular for transient or linear for stable transfection), solublised in 50µl sterile dH_2O, was gently mixed with the cell suspension and placed into a 0.4cm cuvette. The cells were electroporated as described (Table 2.7.1) and left to stand at room temperature for 10 minutes before being plated at $2x10^4$/ml.

All other cell lines were electroporated using the BioRad pulse electroporator. In this system, cells were resuspended in the appropriate buffer and immediately mixed with the desired quantity of DNA in a 0.4cm cuvette (Table 2.7.1). Following electroporation, cells were left to stand at room temperature for 10 minutes before being plated (section 2.4).

2.7.3 Production of Experimental Cell Lines

In the case of transient expression, integrated genes were usually reporter proteins, i.e. green fluorescent protein (GFP). In these cases cells were analysed directly 48 hours after transfection. However, in the case of stable integrated transgenes, cells were left for 48 hours after transfection before applying drug selection at the desired concentration (Table 2.7.2) previously determined by performing a killing curve. Each transfection experiment included a non-selection control and a no DNA control, to indicate a) that the integration of the DNA had not resulted in promotion of cell death, and b) that the selection regime was working, respectively.

Generally, after two weeks in drug selection, discrete colonies of cells had formed and were large enough to pick, if a clonal cell line was required. To pick individual colonies, the media was removed and ~10µl of TEG was taken up into a 200µl sterile filter-tip. This was then used to collect and gently disaggregate each individual colony, which was then transferred to an individual well of a 48-well plate. The clones were then expanded in culture as previously described (section 2.4) into 1 well of a 6-well plate and frozen into two 0.5ml samples, to form the basis of an

individual cell line. If a pooled population was the desired outcome, after drug selection had killed all of the cells on the no-DNA control plate, the whole plate was TEG treated and passaged together to be expanded before freezing (section 2.6).

Cell Line	Species	Selection	Features/Comments	Source
M2	Human	200µg/ml G418	Originally H9 cells. Have hTERT driven α1,3gal transgene randomly integrated. Strong expressing clone with low levels position effect variegation.	Dr H. Priddle
F11	Human	200µg/ml G418	Originally H9 cells. Have hTERT driven α1,3gal transgene randomly integrated. Weakly expressing clone with low levels position effect variegation.	Dr H. Priddle
Egal-9	Human	200µg/ml G418	Originally H9 cells. Have EF1α driven α1,3gal transgene randomly integrated. Pooled cell population.	Zoë Hewitt
Egal-293	Human	500µg/ml G418	Originally HEK 293 cells. Have EF1α driven α1,3gal transgene randomly integrated. Pooled cell population.	Zoë Hewitt
H7-K^k	Human	200µg/ml G418	Originally H7 cells. Pooled cell population which has a hTERT driven H2-K^k transgene randomly integrated.	Zoë Hewitt
H7-K^k/β2	Human	200µg/ml G418	Originally H7 cells. Co-electroporated the hTERT-H2-K^k construct with one carrying a constitutive mouse β-2 Microglobulin gene (Prof. S Ono, University college London), pooled population.	Zoë Hewitt
Oct-4/GFP-6	Human	200µg/ml G418	Originally H9 cells. Have 8.5Kb mouse Oct-4 driven GFP transgene randomly integrated. Strong expressing clone with low levels position effect variegation.	Zoë Hewitt

Table 2.7.2: Stable Transgenic Cell Lines Produced and Used in this Study.

2.8 Characterisation of Normality and Pluripotentiality of Human ES cells

2.8.1 Cytogenetic Analysis

2.8.1.1 Preparation of Mitotic Spreads

To prepare chromosomes from human ES cells a confluent $25cm^2$ flask was split between three $25cm^2$ flasks and allowed to proliferate for 25 hours. At 25-hours colcemid (Gibco) was added to the cell culture media at 10µl/ml, to destroy the mitotic spindle, and cells were incubated under normal growth conditions for 2 hours. At 27-hours the cells were harvested, cell culture media was decanted and the cells were washed with PBS, which was decanted into the culture supernatant and saved. TEG was used to disaggregate the cells, 4 minutes at 37°C, before adding back the decanted culture supernatant to inactivate it. The cells were collected by centrifugation at 260g for 5 minutes, after which, the supernatant was discarded and cell pellet re-suspended, by vortexing, in 8mls of hypotonic solution. Incubation in hypotonic solution was exactly 10 minutes at room temperature, to prevent over swelling of cells, after which the cells were pelleted as above.

Cells were resuspended in fixative (3:1 ratio of methanol/acetic acid) by vortexing at room temperature. It was essential that the first 2-3mls of the first fixation only, be added drop-by-drop down the side of a tilted tube slowly. The final volume was 8ml. After the first fixation, the cells were then re-pelleted as before, and a further two rounds of fixation were applied. After the final centrifugation, the supernatant was discarded and the cells re-suspended by gently tapping the side of the tube. A small

amount of fix was then added to thin the cell suspension; the aim was to get a slightly cloudy solution to give the best cell density.

A single drop of the chromosome preparation was then placed onto an ethanol cleaned and polished microscope slide; the slide was moved in a circular manner whilst tilted, to encourage spreading, and allowed to air dry. The mitotic index was assessed by phase contrast microscopy, prior to performing chromosome banding.

2.8.1.2 G-Banding and Karyotype Analysis

Giemsa-banding was performed 72 to 96 hours after the preparation of mitotic spreads. The slide preparations were incubated in 40ml 2X SSC at 65°C for 2-4 hours. Subsequently, the slides were rinsed gently in cold running tap water and then placed in 0.25% Bacto-trypsin (Biotrace Fred Baker ltd) for between 10 and 20 seconds. The period of exposure with trypsin is critical, over exposure results in "fluffy" chromosomes which cannot be karyotyped. Following treatment with trypsin, the cold-water wash was repeated, before placing slides in Giemsa, diluted 1:20 in Gurrs R66 phosphate buffer (Gibco) pH6.8, for 10 minutes at room temperature. Slides were then rinsed gently in running cold water, blotted dry and placed in xylene (within a fume cupboard) for 5 minutes, before mounting in PERTEX mounting medium (CellPath).

The chromosome-banding pattern was then visualised under a x100 oil objective, photographed and the karyotype analysed by Judith Fletcher (Roslin Institute,

Edinburgh) in accordance with the International Standard for Cytogenetic Nomenclature (ISCN).

2.8.2 Flow Cytometry Analysis of Characteristic ES Cell Surface Markers

Human ES cells were harvested as appropriate, and disaggregated into a single cell suspension in KO-DMEM. The cells were counted (section 2.5) and used at a density of $1x10^6$ per ml. The cell suspension was dispensed into 1ml aliquots across 9 pre-labelled 5ml polystyrene tubes (Falcon). The tubes were centrifuged at 260g for 5minutes and the supernatant was discarded. The cells were then blocked by re-suspending the cell pellet in 50µl of 40% heat inactivated rabbit serum (blocking buffer), to prevent any non-specific binding of the antibody, for 15minutes on ice. Primary antibodies were diluted as appropriate (Table 2.8.1) in PBS plus 10% FBS and 2mM EDTA (staining buffer) and antibody or staining buffer added to the appropriate tube (final volume 100µl). Cells were incubated in the presence of the primary antibody for 30 minutes on ice, after which the unbound antibody was removed by washing twice with 3ml of staining buffer followed by centrifugation at 260g for 5 minutes.

Following washing, the cell pellet was resuspended in 100µl of appropriately diluted secondary antibody or pre-conjugated primary antibody, in staining buffer (Table 2.8.1). The cells were incubated in the presence of the fluorescent conjugated antibody for 30 minutes on ice in the dark. The unbound antibody was removed as before, except that the wash was performed with 1xPBS alone. The wash supernatant was discarded and the cell pellet resuspended in 0.5-1ml PBS or 0.1%

paraformaldehyde (PFA) in PBS if the samples were to be stored. Stored samples were kept at 4°C in darkness for no more than 7 days, before acquisition of data.

ES Marker Primary Antibody Dilution		**Isotype Control Dilution**		**Secondary Antibody Dilutions** (Southern Biotechnologies)	
SSEA-4 (50µg/ml) (Development studies Hybridoma Bank)	1/3	IgG_3 (1.0mg/ml, Sigma)	1/3	FITC-IgG_3 (0.5mg/ml)	1/100
SSEA-1 (40µg/ml) (Development studies Hybridoma Bank)	1/5	IgM (1.0mg/ml, Sigma)	1/5	R-PE-IgM (0.25mg/ml)	1/100
Tra-1-60 (1.18mg/ml) (Chemicon)	1/10	IgM (1.0mg/ml, Sigma)	1/10	R-PE-IgM (0.25mg/ml)	1/100
Tra-1-81 (1.51mg/ml) (Chemicon)	1/20	IgM (1.0mg/ml, Sigma)	1/20	R-PE-IgM (0.25mg/ml)	1/100
R-PE-CD9 (50µg/ml) (BD Pharmingen)	1/5	R-PE-IgG_1 (6.25µg/ml) (BD Pharmingen)	1/5	Pre-conjugate	

Table 2.8.1 Specific antibody conditions for the analysis of characteristic cell surface markers of undifferentiated human ES cells (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998)

Samples were analysed with a FACScan flow cytometer (Beckton Dickenson Immunocytometry Systems (BD), UK) equipped with an air-cooled argon ion laser emitting 15mW of blue light at 488nm and with standard filter setup. Detection of undifferentiated human ES cells used the parameter settings outlined in Table 2.8.2. Simultaneous measurements of forward light scatter (FSC, relative size), 90 degree light scatter (SSC, internal complexity), and green fluorescence from FITC-stained cells or red fluorescence from R-PE-stained cells, (detected through a 530-nm, 30nm-bandwidth band-pass filter (FL1 channel) or 585-nm, 42-nm bandwidth band-pass filter (FL2 channel)) were collected. The FSC parameter was used as the threshold and set at a value of 52. All cell analyses were performed using single colour staining, at the high rate setting (60µl +/- 7µl/min), using FACSFlow solution

(BD) as the sheath fluid, recording at least 40,000 events. Data were collected in list mode as pulse height signals (4 decades each on a logarithmic scale) and analysed using CellQuest Pro software (BD).

Detector	Human ES cells		HEK 293 Cells		K562 Cells		U937 Cells		Mode
	Volt	Amp Gain	Volt	Amp Gain	Volt	Amp Gain	Volt	Amp Gain	
FSC	E00	1.50	E-1	6.06	E-1	7.00	E-1	7.00	Linear
SSC	340	1.00	350	1.00	370	1.00	370	1.00	Linear
FL1	600	1.00	570	1.00	600	1.00	640	1.00	Logarithmic
FL2	435	1.00	460	1.00	480	1.00	430	1.00	Logarithmic

Table 2.8.2: BD FACScan acquisition parameters for human ES, HEK 293, K562 and U937 cells.

Using the same protocol, flow cytometry was used further in Chapter 3 to determine the level of expression and variegation of clones containing the α(1,3)Gal transgene and also to determine their ability to bind natural anti-α-gal antibodies from human serum (Table 2.8.3). In Chapter 4, flow cytometry was use to assess the levels of complement inhibitor proteins (Table 2.8.3) on a number of cell types (for parameter settings see Table 2.8.2), and also as a method of titrating blocking antibodies for use in inhibition complement-mediated lysis. Finally, in Chapter 7, regulation of the ES cell surface markers (Table 2.8.1) excluding CD9 but including the α-gal epitope and GD2 (Table 2.8.3), were assessed longitudinally following differentiation (section 2.10.2.2).

Epitope	Primary antibody dilution	Isotype	Secondary antibody or Fluorochrome	Supplier
α-gal (200μg/ml)	1:40	Isolectin *BS-IB*$_4$ (see 2.9.4.2)	FITC-Conjugated	Sigma
MCP (CD46) (0.1mg/ml)	1:10	IgG1 (0.5mg/ml)	FITC-Conjugated	Serotech
DAF (CD55) (0.2mg/ml)	1:10	IgG2a (0.2mg/ml)	R-PE-Conjugated	BD Pharmingen
MIRL (CD59) (0.5mg/ml)	1:10	IgG2a (0.5mg/ml)	FITC-Conjugated	BD Pharmingen
HD3 (CD55) (1.5mg/ml)	1:250	IgG1 (0.5mg/ml)	Goat anti-mouse IgG-FITC (1.0mg/ml, Sigma)	Gifted by Prof P Morgan
Human Ig (1.0mg/ml)	1:50	IgG, IgM, IgA and IgE	Goat anti-mouse IgG-FITC (1.0mg/ml, Sigma)	Southern Biotech
MEM43 (CD59) (0.6mg/ml)	1:250	IgG2a (0.5mg/ml)	Goat anti-mouse IgG-FITC (1.0mg/ml, Sigma)	Gifted by Prof P Morgan
GD2 (VIN2PB22) (50μg/ml)	1:3	IgM (1.0mg/ml)	Goat anti-mouse IgM-R-PE (0.2mg/ml Caltag)	Developmental Studies Hybridmoa bank

Table 2.8.3: Antibodies and their conditions for use in flow cytometry experiments described in Chapters 3, 4 and 7.

2.8.3 Alkaline Phosphatase Staining of Human ES Cells

The presence of alkaline phosphatase was determined using the BCIP/NBT alkaline phosphatase substrate kit IV, supplied by Vector Labs in accordance with the instructions provided. Culture medium was removed and the cells were washed with PBS before being fixed with 4% PFA for 20 minutes at room temperature. The fixative was then removed and the cells washed twice with PBS. The BCIP/NBT working solution was prepared immediately prior to use, by mixing 2 drops of each of the reagents 1, 2 and 3 in 5mls of 100mM Tris-HCl buffer at pH 9.5. Enough of the working stock to cover the surface of the cells (0.5-1.0ml) was added and they were then incubated in the dark for 1 hour, after which the stain was removed and the

cells were washed with distilled water. Presence of alkaline phosphatase produced a blue stain, which was visualised by eye, or using differential interference contrast microscopy (DIC) and photographed.

2.8.4 *In vitro* Differentiation of Human ES Cells – Undirected

The first stage of *in vitro* differentiation was the formation of 3-D cellular structures known as embryoid bodies (EB). ES cells culture with a TEG passaging regime were disaggregated into a single cell suspension and reseeded onto non-adherent culture plastic in CM. The cells were maintained in this way for 2 days, and spontaneously clumped together to form small cell aggregates. After 2 days, the cell aggregates were transferred to basic differentiation medium (basic human ES medium, un-conditioned, minus hbFGF with 10% FBS replacing the 20% KO-Serum Replacement) and maintained in suspension for a further 5 days before being plated on 0.1% gelatin-coated SonicSeal Bucket Slides (NUNC), at a density of 5-10 EB's per well. During the five days suspended in basic differentiation medium, the EB's first became very dense, and showed signs of a defined membrane before becoming vacuolated immediately prior to plating. Once the EB's had been transferred to gelatin-coated plates, they grew out very quickly and began to differentiate into numerous cell types. Cultures were maintained in this way for 14-21 days with medium changed every 48-72 hours.

2.8.4.1 Analysis of Differentiated Cell Types by Immunohistochemistry

Cells that had been differentiated for 14 - 21 days were washed with PBS and fixed in 4% PFA for 20 minutes at room temperature. After fixation the cells were washed

twice in PBS and permeabilised with absolute ethanol for 2 minutes at room temperature. The PBS wash was repeated following permeablisation and the cells incubated with 10% normal goat serum at room temperature for 1 hour to block non-specific binding of the antibodies. Primary antibodies were diluted as required (Table 2.8.4) in 1% normal goat serum. Blocking serum was removed and cells were incubated with 200µl of primary antibody for 2 hours at room temperature, while shaking slowly. After 2 hours the primary antibodies were removed and the cells washed three times for 5 minutes in PBS.

Germ Layer	Primary antibody	Dilution	Supplier
Mesoderm	Muscle Actin (0.1mg/ml)	1/50	Dako
Ectoderm	β-Tubulin (4.6mg/ml)	1/1000	Sigma
Endoderm	α-fetoprotein (9.2mg/ml)	1/500	Sigma

Table 2.8.4: Antibody conditions for markers routinely used to assess the *in vitro* differentiation ability of undifferentiated human ES cells.

The secondary antibody (FITC labelled Goat anti-mouse-IgG (1.0mg/ml, Sigma)) was diluted 1/200 in 1% normal goat serum and the cells were incubated with 200µl for 30 minutes at room temperature, shaking gently in the dark. After incubation with the secondary antibody, the cells were washed three times for 5 minutes in PBS. The wells were then mounted in Vectashield plus Dapi (Vecta Labs) and sealed with pang (Truflex) and allowed to dry. Slides were stored at 4°C in the dark until viewed with a fluorescent microscope.

2.8.5 *In vitro* Osteogenic Differentiation of Human ES cells

Directed differentiation of human ES cells towards the osteogenic lineage was performed using an adaptation of the protocol published by Sottile *et al.,* (2003). Briefly, differentiation was initiated by the formation of EB's (section 2.8.3), maintained in suspension for a total of 4 days, 2 days in CM and 2 days in basic differentiation media. After 4 days in suspension the EB's were disaggregated with TEG into a single cell suspension, counted (section 2.5) and plated onto 0.1% gelatin coated tissue culture plastic (NUNC) at a density of $1x10^5$/ml in basic differentiation medium, in a humidified incubator with 5% CO_2, overnight at 37°C. The following day, cells were either maintained in basic differentiation medium (OS-), or transferred to differentiation medium supplemented with 10mM β-glycerophosphate 50μM ascorbic acid, and 0.1μM dexamethasone as osteogenic factors (OS+) for a total of 20 days. Culture medium was replenished every 48-72 hours, and the progress of osteogenic differentiation assessed at 0, 3, 6, 9, 13, 16 and 20 days post induction, by Alizarin Red S staining and calcium deposition.

2.8.5.1 Analysis of Osteogenic differentiation

Alizarin-red S Staining

Alizarin-red S combines with calcium atoms to form a bright red stain, commonly used in the detection of mineralising nodules (Sottile *et al.,* 2003). Cells were fixed at the desired time point (2.8.5) by removing the culture medium, washing twice with PBS and incubating with 4% PFA at 4°C for 10 minutes. Following fixation the PFA was removed and the cells were washed once with distilled water. Care was taken not to dislodge the monolayer. Alizarin-red S (Sigma) stock solution was prepared

as a 1% solution in distilled water and filtered through 3MM Whatman filter paper before use. The fixed cells were incubated with sufficient Alizarin-red S solution to cover the cells (0.5-1ml) at room temperature for 10 minutes. Following the staining, the cells were washed, carefully, three times with distilled water and photographed immediately.

Calcium Deposition

The purpose of this assay was to determine the matrix-associated mineralization following treatment with osteogenic factors. Cells were fixed at the desired time point (see section 2.8.5) by removing the culture medium and washing three times with 0.9% sodium chloride. The cells were left to dry completely at 37°C, before lysing them with 50µl 0.1M sodium hydroxide for 2-3 hours. The lysis buffer was neutralised by incubation with 20µl of 2M hydrochloric acid and the plates dried in a 60°C incubator. Once completely dry, the plates were assessed for calcium deposition, using a calcium colorimetic kit obtained from Randox, using the manufacturer's instructions as guidelines. Briefly, matrix associated calcium deposition from differentiated human ES cells was established against a standard curve produced by diluting the standard calcium solution (2.5mM/l) provided in the Randox kit. To 25µl of each diluted standard, ranging from 0-100µM in distilled water, and to each sample well, 200µl of assay buffer was added and incubated at room temperature for 10 minutes. During this time calcium ions form violet complexes with O-Cresolphthalein complexone (the active component of the kit) in the alkaline medium. The absorbance of the sample and the standard were measured immediately using a spectrophotometer at a wavelength of 570nm.

2.8.6 *In vivo* Differentiation of Human ES Cells

For human ES cells, evidence of continued pluripotentiality can only be achieved *in vivo* by the formation of teratomas. To this end, $1x10^7$ undifferentiated human ES cells suspended in PBS were injected intramuscularly, (under license by Dr J. McWhir, Roslin Institute, Edinburgh) into the hind thigh of severe combined immunodeficient (SCID) mice strain C.B-17/Icr (Harlan UK Ltd). Generally, tumours took between 3 and 5 months to develop, after which the mice were sacrificed and the tumours removed. Tumours were fixed whole, or cut into segments if very large, with 4% PFA for 20 minutes at room temperature and subsequently stored at 4°C in sterile PBS before being analysed.

2.8.6.1 Analysis of Tumour Sections

Paraffin wax embedding

Tumours were embedded in paraffin wax in an automated system using a Shandon Hypercentre XP processor (Shandon Scientific UK Ltd). Details of the process are detailed in Table 2.8.5. Once embedded, tumours were cut into 10µm sections using a Microm HM325 rotary microtome (MICROM international GmbH).

The whole tumour was sectioned, with 3 sections every 10 being transferred to polylysine coated microscope slides. The slides were allowed to dry at least overnight at room temperature before being stained.

Step	Solution	Concentration (%)	Time (mins)	Temperature (°C)
1	Ethanol	70	90	Room Temp
2	Ethanol	96	90	Room Temp
3	Ethanol	96	90	Room Temp
4	Ethanol	100	90	Room Temp
5	Ethanol	100	90	Room Temp
6	Ethanol/Paraffin clearing agent	50/50	90	Room Temp
7	Ethanol/Paraffin clearing agent	50/50	60	Room Temp
8	Paraffin clearing agent	100	60	Room Temp
9	Paraffin clearing agent	100	60	Room Temp
10	Paraffin clearing agent	100	60	Room Temp
11	Paraffin clearing agent	100	60	Room Temp
12	Paraffin Wax	100	60	60
13	Paraffin Wax	100	60	60

Table 2.8.5: Automated process for the paraffin wax embedding of tumours using a Shandon Hypercentre XP processor (Shandon Scientific UK Ltd)

Haematoxylin and Eosin (H&E) Staining

Before staining, the dried slides were de-waxed in xylene (BHD) for 5 minutes before being slowly rehydrated in serial ethanol incubations, at successively reduced concentrations (2 minutes at 75% followed by 2 minutes at 65%) before being incubate in water for a further 2 minutes. Sections were then stained with haematoxylin (Sigma) for 5 minutes and washed under running water for approximately 5 minutes, until the sections were blue rather than deep purple in colour. Sections were further stained with eosin (Sigma) for 2-5 minutes and excess stain removed by washing in water for 2 minutes. Sections were then dehydrated in successively increasing concentrations of ethanol (1 minute at 65% followed by 1 minute at 75%). Slides were then incubated in xylene (in a fume cupboard) for 5 minutes to remove traces of ethanol and allowed to dry slightly before mounting the slides with PERTEX mounting medium (CellPath). For the best results, slides were

left overnight to dry completely. Histological analysis, by light microscopy, of the tumour sections was performed by Dr David Brownstein (Research Animal Pathology Core Laboratory, University of Edinburgh, UK).

2.9 Analysis of Gene Expression

2.9.1 Preparation of RNA

RNA was prepared using the RNAeasy system from Qiagen, in accordance with the manufacturers protocol. Cells grown as monolayers on tissue culture plastic ($25cm^2$) were directly lysed with 600µl of buffer RLT. The viscous cell lysate was homogenised on a QIAshredder column held in a 2ml collection tube, and microcentrifuging it at 10,000g for 2 minutes. To the flow-through, an equal volume of ethanol (70% made in RNase-free water) was added and subsequently transferred in two 600µl quantities to an RNAeasy column (Qiagen) and microcentrifuged at 8000g, to bind the RNA. The flow-through was subsequently discarded and the column washed with 700µl of buffer RW1 (spun 8000g for 15 seconds). The RNAeasy column was then transferred to a fresh collection tube and washed a further 2 times with 500µl of buffer RPE. Following the third wash, the flow-through was discarded and the column microcentrifuged for 1 minute to dry the silica-gel membrane and remove carry-over of buffer RPE. The RNA sample was then eluted into a fresh 1.5ml collection tube by adding 30-50µl of RNase-free water to the membrane and microcentrifuging at 8000g for 1 minute. The elution step was repeated twice to improve the RNA yield. Typically the final volume was 80µl.

2.9.1.1 Assessing the Quality and Quantity on RNA

Spectrophotometric measurements were used to determine the quantity and quality of the RNA preparations. Typically an RNA sample was diluted 1:50 with DECP-treated water (Sigma) and the absorbance read at 260nm to give the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 40µg/ml of

RNA. The ratio between readings at 260nm and 280nm provided an estimation of the purity of the RNA preparation. Pure RNA has an OD_{260}/OD_{280} ratio of between 1.9 and 2.1.

RNA integrity was also examined crudely by agarose gel electrophoresis. The presence of 2 ribosomal bands representing the 18S (1.9kb) and 28S (5.0kb) components of RNA should appear as discrete sharp bands, with the intensity of the 28S band being twice that of the 18S band. If the ribosomal bands produced a smear of smaller sized RNAs, it was considered likely that the RNA had undergone major degradation during preparation and was discarded.

2.9.2 cDNA synthesis and Reverse Transcriptase (RT) PCR

First strand cDNA synthesis and PCR were performed simultaneously using the Superscript One-Step RT-PCR system from Invitrogen and gene specific primers (Table 2.9.1). This system contains both Superscript II reverse transcriptase, a modified version of Moloney Murine Leukemia Virus (M-MLV) RT, engineered to reduce RNase H activity and increase thermal stability, and Platinum *Taq* DNA polymerase complexed with a proprietary antibody that inhibits polymerase activity at ambient temperatures. Together these enzymes permit both cDNA synthesis and PCR in a single reaction, due to the inactivation of superscript II RT and activation of Platinum *Taq* during the 94°C denaturation step in PCR cycling, providing a "hot start" for PCR resulting in increased primer specificity.

A typical RT-PCR reaction consisted of 125ng of sample RNA, mixed with 0.2μM of both the sense and anti-sense primers, 6.25μl of 2X reaction mix, containing 0.4mM of each dNTP and 2.4mM $MgSO_4$, and 0.25μl of RT/Platinum *Taq* Mix, made up to a final volume of 12.5μl with autoclaved DEPC treated RNase-free distilled water.

Gene	Primers	Product Size		Annealing temp.
		cDNA	gDNA	
Human specific β-Actin	Fwd: GCCACGGCTGCTTCCAGC Rev: CAAGATGAGATTGGCATGGCT	528bp	735bp	59°C
Mouse Specific β-Actin	Fwd: CGTGGGCCGCCCTAGGCACCA Rev: CCCCCCTGAACCCTAAGGCCAA	242bp	330bp	59°C
Mouse Specific H2-K^k	Fwd: CTGGCTCCGACTCAGACC Rev: GCGCTGATCACCAAACACAA	461bp	837bp	59°C
Mouse Specific β-2 Microglobulin	Fwd: GGTGCTTGTCTCACTGACC Rev: CCAGTATGGCCGAGCCCA	302bp	4111bp	59°C
Human Specific β-2 Microglobulin	Fwd: GCTCCGTGGCCTTAGCTG Rev: CATTCAGACTTGTCTTTCAGC	224bp	4033bp	59°C

Table 2.9.1 Species specific RT-PCR primers for the β-actin, H2-K^k and β-2 microglobulin genes. Primers (MWG) were designed to cross introns so that genomic contamination was easily detected. The table provides the product sizes for both cDNA and gDNA and the annealing temperature of the specific primers.

A standard set of PCR cycling conditions were used for each of the primer sets, on an MJ Gradient 4 block PCR machine. cDNA synthesis was achieved in a single cycle of incubation at 50°C for 30 minutes followed by pre-denaturation (inactivation of Superscript II and activation of Platinum *Taq*) at 94°C for 2 minutes. PCR amplification started immediately, with 40 cycles of denaturation at 94°C for 15 seconds, primer annealing at optimal temperature (Table 2.9.1) for 60 seconds, followed by a 1 minute extension time at 72°C. A final extension period of 10 minutes at 72°C was incorporated before holding at 4°C.

PCR products were resolved by horizontal gel electrophoresis on 2% (w/v) agarose gels, run at 80V for 2 hours (section 2.3.2).

2.9.3 Western Blotting

Expression of the H2-K^k protein was assessed in Chapter 3 by Western blot.

2.9.3.1 Protein Isolation

Cells grown (see section 2.4) to confluence in a $25cm^2$ tissue culture flask were used as a source of protein. Culture medium was removed and the cells washed once with sterile PBS. Cells were then harvested from the tissue culture plastic (NUNC) mechanically using sterile cell scrapers (Greiner Bio-one) into PBS and collected by centrifugation at 260g for 5 minutes. The cell pellet was then resuspended in 200µl of protein lysis buffer containing 1% Triton-X 100 and protease inhibitors (Complete mini cocktail, Roche) on ice for 30 minutes. Following incubation on ice, the lysate was centrifuged at 10,000g in a microcentrifuge at 4°C for 5 minutes. The supernatant was then collected and dispensed into 20µl ampoules and snap frozen on dry ice before being transferred to –80°C for long term storage.

2.9.3.2 SDS Polyacrylamide Gel Electroporesis

Protein lysates were thawed on ice and mixed with an equal volume of 2x SDS loading buffer before being loaded (10-20µl) onto a pre-cast Tris-glycine gel (Invitrogen) using the XCell SureLock mini-cell apparatus (Invitrogen). Normally

the proteins were run under reducing condition, 200mM DTT was present in the loading buffer and samples were denatured at 99.9°C for 5 minutes prior to loading. In Chapter 3, the detection of H2-K^k, was attempted using non-reducing conditions which involved leaving out DTT from the loading buffer. The samples were separated by electrophoresis at 100V for 2 hours using 1x Tris-glycine running buffer.

2.9.3.3 Western Transfer

Using the XCell SureLock blotting module (Invitrogen) a sandwich consisting of two blotting pads, one piece of 3MM paper, a piece of polyvinylidene difluoride membrane (PVDF: Hypond-P, Amersham Pharmacia) the Tris-glycine gel, a second piece of 3MM paper and two further blotting pads was constructed. All components were pre-soaked in 1X western transfer buffer, with the exception of the hydrophobic PVDF membrane which was first immersed in methanol and equilibrated in distilled water prior to exposure to transfer buffer. The sandwich was then placed into the blot module and transfer was allowed to proceed for 1 hour 30 minutes at 100mA. Following transfer the sandwich was disassembled and the PDVF membranes blocked with 5% milk protein for 2 hours at room temperature with agitation. Membranes were then either immunoblotted immediately or transferred to PBS containing 0.1% Tween20 (wash solution) and stored at 4°C.

2.9.3.4 Immunoblotting

Primary antibodies were diluted (Table 2.9.2) in freshly made blocking solution and incubated overnight at 4°C with gentle agitation. Membranes were then washed 6 times for 10 minutes each in wash solution at room temperature. HRP-conjugated secondary antibody was then diluted appropriately (Table 2.9.2) in fresh blocking solution, applied to the membrane and incubated at room temperature for 2 hours with gentle agitation. Following incubation with the secondary antibody, the wash step was repeated, (6 washes of 10 minutes). Membranes were then exposed to pre-mixed ECL western agents (Amersham Pharmacia), wrapped in Saranwrap (Saran) and exposed to film (Amersham Pharmacia) for 30 seconds to 15 minutes. Film was developed in a Konica SRX-101A X-ograph machine.

Primary Antibody	Dilution	Source	Secondary dilution	Source
Anti-β-Actin (0.2mg/ml)	1:250	Santa Cruz	HRP-anti-goat (2.0mg/ml) 1:500	Autogen Bioclear
H2-K^k (0.5mg/ml)	1:50-1:250	Southern Biotechnology	HRP-anti-mouse (0.73mg/ml) 1:500	Amersham Pharmacia

Table 2.9.2 Antibody dilutions for the detection of H2-K^k and β-actin by Western Blot.

2.9.4 Immunochemistry

2.9.4.1 Antibody Staining

Cells were plated on a relevant extracellular matrix in 4-welled SonicSealed Bucket slides (NUNC) or on glass cover slips and grown under normal conditions (2.4) until they reached a suitable level of confluence (60-70%). Culture medium was removed and the wells washed with PBS. If pre-fixation was necessary, (Table 2.9.3) cells

were fixed in 4% PFA at room temperature for 20 minutes. After fixation, PFA was removed and the cells were then washed twice with PBS. For internal epitopes, permeablisation of the cell membrane is required, this if necessary (Table 2.8.1) was achieved through incubation with absolute ethanol for 2 minutes at room temperature. Ethanol was promptly removed and the cells washed twice with PBS.

Whether fixed, permeablised or stained live, non-specific binding of the primary antibody was blocked by incubation in normal serum, specific to the species that the secondary antibody was raised in, typically 5-10% normal goat serum (Table 2.9.3), for 30 minutes at room temperature. After blocking non-specific activity, the blocking agent was removed and cells were incubated with primary antibody, diluted as specified (Table 2.9.3) in 1% blocking agent, for 1 hour at room temperature. Cells were then washed with PBS at room temperature three times for 5 minutes each, before being incubated with 200µl of secondary antibody diluted appropriately as indicated in Table 2.9.3, for 1 hour in the dark. If the primary antibody was pre-conjugated, the cells were incubated with this diluted appropriately for 1 hour (Table 2.9.3). Following incubation with the secondary antibody, the cells were washed again with PBS three times for 5 minutes each at room temperature, in the dark. After the final wash the cells were blotted dry and mounted with 1 drop of Vectashield with Dapi (Vecta Labs), to stain the nuclei, with a glass cover slip on top and sealed with Pang (Truflex). The slides were allowed to dry completely overnight at 4°C before being viewed with fluorescence microscopy.

Epitope	Fix	Perm	Block	Antibody Dilutions	
				Primary	Secondary
Beta-2 Microglobulin	Post-fix 1% PFA	No	5% Heat inactive Goat Serum	β-2 M (0.2mg/ml) 1/100 Santa Cruz	Goat anti-mouse IgG2b-R-PE (0.4mg/ml) 1/200 Santa Cruz
H2-K^k	Post-fix 1% PFA	No	5% Heat inactive Goat Serum	H2-K^k (0.5mg/ml) 1/50 BD Pharmingen	Goat anti-mouse IgG Whole Molecule-FITC (1.0mg/ml) 1/200 Sigma
H2-K^k	Post-fix 1% PFA	No	5% Heat inactive Goat Serum	H2-K^k-FITC (0.5mg/ml) 1/100 Southern Biotech	
Oct-4	Pre-Fix 4% PFA	Yes	10% Goat Serum	Oct-4 (0.2mg/ml) 1/200 Santa Cruz	Goat anti-mouse IgG Whole Molecule-FITC (1.0mg/ml) 1/200 Sigma
SSEA-3	Pre-fix 4% PFA	No	10% Rabbit Serum	SSEA-3 (50µg/ml) 1/100 Development studies Hybridoma Bank	Rabbit anti-mouse IgM Whole Molecule-FITC (4.8mg/ml) 1/200 Sigma
SSEA-4	Pre-fix 4% PFA	No	10% Goat Serum	SSEA-4 (50µg/ml) 1/100 Development studies Hybridoma Bank	Goat anti-mouse IgG Whole Molecule-FITC (1.0mg/ml) 1/200 Sigma
TRA-1-81	Post-fix 1% PFA	No	10% Goat Serum	Tra-1-81 (1.51mg/ml) 1/20 Chemicon	Goat anti-mouse IgM-R-PE 1/50 Caltag

Table 2.9.3: Specific conditions required for the detection of epitopes by antibody-mediated immunochemistry.

2.9.4.2 Bandeiraea simplicifolia isolectin B4 (BS-IB4,) Staining

Cell surface expression of the α-gal epitope was assessed using a fluorescein isothiocyanate (FITC)-conjugated *Bandeiraea simplicifolia* isolectin B4 (*BS-IB4*, Sigma) specific for α-gal epitope with an adapted protocol from Xing *et* al (2001). Briefly, after removing the culture medium, cells were washed in sterile complete PBS (containing Mg^{2+}/Ca^{2+}) supplemented with 1% BSA (Gibco) three times for 5 minutes each. The cells were then incubated with the 5µg/ml *BS-IB4* lectin,

dissolved in complete PBS plus 10% newborn calf serum (NCS), at 4°C for 90 minutes in the dark. For flow cytometry, *BS-IB$_4$* was dissolved in normal PBS (without Mg^{2+}/Ca^{2+}) to encourage a single cell suspension, for immunochemistry, Mg^{2+}/Ca^{2+} was added to prevent the cells from detaching. The wash step described above was repeated a further 3 times, and the cells post-fixed in 1% formaldehyde at room temperature for 20 minutes. The fix was removed and the cells washed and store, if necessary, in PBS at 4°C.

2.9.4.3 Giemsa Staining

Giemsa is a deposit stain that stains DNA. Culture media was removed and cells washed once with PBS. Cells were then fixed with 100% methanol for 20 minutes at room temperature. The methanol fix was then removed and the cells incubated with 5% Giemsa, diluted in either Gurrs buffer pH6.5 or PBS pH7, for between 15 and 30 minutes at room temperature. After the stain had been removed, cells were washed 3-5 times with slow-running water. When Giemsa was being used to provide images with morphology, the cells were placed in water and the images taken immediately, after which the plates were dried and kept in the dark before whole plate photography was undertaken.

2.10 Strategies for the Selective Elimination of Human ES Cells

2.10.1 Complement Mediated Cell Lysis

2.10.1.1 The Calcein-Release Assay

Complement-mediated lysis was determined using the Calcein-release assay as a fluorescent equivalent to the ^{51}Cr-release assay, described by B. Spiller in Complement Methods and Protocols (2000). Calcein is an organic, polyanionic fluorochrome derived from fluorescein. In its acetoxymethyl ester form (Calcein.AM) Calcein is only weakly fluorescent and its non-polar nature allows it to freely diffuse across membranes. However, once inside a living cell, the acetoxymethyl group is cleaved by ubiquitous, non-specific cytoplasmic esterases, changing the nature of the molecule from non-polar to polar and thus trapping it inside the cell until the cell membrane is breached. By measuring the fluorescence of the supernatant after exposure to complement and calculating it as a percentage of the total fluorescence of the original cell aliquot, it is possible to determine the level of cell lysis as a result of complement exposure in terms of % Calcein-release. Specific lysis can be calculated by subtracting the % spontaneous Calcein-released from control cells that were not exposed to complement.

2.10.1.1.1 Pre-loading with Calcein.AM

To determine the optimal concentration of Calcein.AM for labelling, cells lines were harvested with TEG (section 2.4), counted (section 2.5) and re-suspended at a concentration of $2x10^6$/ml in 50µl of culture medium containing 0, 2, 5, 10, 20, 40, 80 and 160µM Calcein.AM. The cell suspensions were then incubated at 37°C with

5% CO_2 in a humidified incubator for 1, 2 or 4 hours in the dark. Excess Calcein.AM was removed by washing the loaded cells twice with KO-DMEM followed by centrifugation at 200g for 5mins. Calcein.AM uptake and Calcein-release were assessed using a 0.1% Triton-X 100 solution to totally lyse the cells, referred to as total release (TR) and normal culture medium to assess the level of spontaneous release (SR). The concentration of Calcein.AM and incubation time at which there was the greatest difference in fluorescence intensity between TR and SR were determined to be the optimal conditions for Calcein.AM labelling (section 2.10.1.1.2 for details of quantifying Calcein-release). For all subsequent experiments cells were loaded with 40μM of Calcein.AM for 2 hours at 37°C with 5% CO_2.

2.10.1.1.2 Quantification of Calcein-Release

Following exposure to complement, cells were pelleted at 200g for 5 minutes. Using the method of Iwanowicz *et al* (2004), a 50μl sample of the supernatant was collected without disturbing the cell pellet, mixed with 50μl of 2x Triton-X 100 and transferred to a black, round bottom 96-well plate. This is referred to as the lysis fraction. Excess supernatant was then discarded and the remaining cells were lysed by re-suspension in 100μl of 0.1% Triton-X 100 and incubated at 37°C with 5% CO_2 in a humidified chamber for 15 minutes. Likewise a 50μl sample of this fraction was collected mixed with 50μl of 2X Triton-X 100 and transferred to a second black, round bottom 96-well plate and is referred to as the detergent fraction. The method of Spiller (2000) differs subtly from that of Iwanowicz *et al.,* (2004) in that Spiller suggested the use of 250μl of both serum and detergent, and the collection of all

250µl to the 96 well-plate, without mixing with 2X Triton-X 100 for the detection of Calcein-release.

The fluorescence in both fractions was then measured using a fluorimeter (Wallac 1420 VICTOR^2) at 530nm emission and 480nm excitation wavelengths and the level of Calcein-release calculated (Figure 2.10.1).

$$\text{\% Calcein Release} = \frac{\text{Intensity of lysis fraction}}{\text{Intensities of the lysis + the detergent fractions}} \times 100$$

$$\text{Specific \% Release} = \text{\% Calcein Release} - \text{\% Calcein Release from the Controls}$$

Figure 2.10.1: Equations for calculating level of lysis in terms of Calcein-release levels

2.10.1.2 Complement-Mediated Lysis through Incubation with Whole Human Serum

2.10.1.2.1 Acquiring Human Serum

Human serum from healthy screened volunteers of blood group-A was either purchased from Harlen Sera Labs (Loughbourgh, UK) as a pooled sample, or collected fresh and pooled from blood group-A healthy volunteers from the Roslin Institute (Edinburgh, UK).

Briefly, 50-100ml of blood was collected from each volunteer by Dr Tim King (Roslin Institute, Edinburgh), separated into 10ml aliquots and allowed to clot at room temperature for 2 hours. The clot was then cut, using a sterile pipette, to

release the serum, and dislodged from the side of the plastic. The clot was then incubated overnight at 4°C to retract and release its serum content. The following day the clot was discarded and the sample was centrifuged at 3220g for 20 minutes at 4°C to remove free red cells. The straw coloured supernatant was collected and either used directly or frozen immediately at -80°C for future use.

2.10.1.2.2 Complement-Mediated Lysis with Endogenous Antibodies

To minimise the effects of plating efficiency, complement-mediated lysis was performed as a suspension assay. Target cells were harvested (see section 2.4), counted (section 2.5) and washed twice with KO-DMEM before being used directly in a lysis experiment or preloaded with Calcein.AM (section 2.10.1.1.1) before use. Cells were collected by centrifugation and resuspended at a density of $1x10^6$/ml in 100µl (10^5) of either active or heat inactivated (HIA) human serum, 0.1% Triton-X 100 or culture medium. Heat inactivation was achieved by incubating the serum in a 56°C water-bath for 30 minutes, mixing regularly to ensure complete inactivation. In an attempt to reduce intra-experimental variation, each data point was represented by 6 replicates and each experiment was repeated independently in triplicate.

The cells were incubated in suspension at 37°C with 5% CO_2 in a humidified incubator for 1 hour, and agitated every 15mins to prevent cells settling. After exposure to complement, the remaining cells were pelleted at 200g for 5 minutes and the supernatant transferred for analysis (section 2.10.1.1.2). Where appropriate the remaining supernatant was removed and the cells resuspended in 0.1% Triton-X 100

to determine the specific % of Calcein released due to complement (section 2.10.1.1.2).

To determine the serum concentration required to achieve the greatest level of lysis, a titration experiment was performed using control PDFF cells, and 0, 2, 5, 10, 20, 50 and 100% serum diluted in appropriate culture medium (section 2.4).

2.10.1.2.3 Complement-Mediated Lysis with Exogenous Antibodies

Complement-mediated lysis using antibodies from an exogenous source was performed using the same approach as for endogenous antibodies (section 2.10.1.2.2), except that the desired antibody was diluted, at its optimal concentration (Table 2.10.1), in the 100µl of human serum in which the cells were resuspended. Incubation with both antibody and serum was then carried out together for 1 hour at 37˚C in a humidified incubator with 5% CO_2.

The exception to this method was when blocking antibodies to CD55 and CD59 were used. In this instance, the cells were incubated in the presence of blocking antibodies, diluted to their optimal concentration (Table 2.10.1) in staining buffer (2% FBS in PBS plus 2mM EDTA), for 15 minutes at room temperature. Excess antibody was then removed by washing the cells twice with KO-DMEM followed by centrifugation at 200g for 5 minutes. At this point the cells were treated as previously outlined (section 2.10.1.2.2).

Antibody Name	Epitope	Dilution	Source
MAB4381 (1.51mg/ml)	Tra-1-81	1/20	Chemicon
HD3 (1.5mg/ml)	CD55	1/250	Kind Gift from Professor Paul Morgan (University of Wales)
MEM 43 (0.6mg/ml)	CD59	1/250	

Table 2.10.1 Exogenous Antibodies used in Complement-Mediated Lysis. TRA-1-81 is endogenously expressed by undifferentiated human ES cells and was used to initiate a complement-mediated attack. CD55 & CD59 are complement regulatory proteins and antibodies were used to inhibit their effects.

2.10.1.3 M2 and HEK 293 Mixed Culture Complement-Mediated Lysis

To determine the sensitivity of the complement-mediated lysis assay, undifferentiated M2 cells were diluted with HEK 293 cells to give a total of $1x10^5$ cells. M2's were spiked into the HEK 293 cultures at 50%, 25%, 12.5%, 6.3%, 3.15%, 1.6%, 0.8% and 0% of the total, in suspension. The serum lysis assay was then performed as previously described (section 2.10.1.2.2), PDFF and H9 pure population respectively were included as positive and negative controls.

Following exposure to active and heat inactivated serum; surviving cells were collected by centrifugation (200g for 5 minutes) and resuspended in KO-DMEM. Cells were seeded in duplicate wells of a 12-well plate, in both ES and HEK 293 culture conditions (section 2.4). Cultures in ES conditions were placed in G418 selection at 500µl/ml so that even rare incidences of ES cell survival could be easily identified. For PDFF, H9, 100% M2 and 100% HEK 293 conditions, a further two duplicate wells of a 12-well plate (active and heat inactivated) were set up so that the initial level of cell lysis could be determined 24 hours after plating by either FITC

conjugated *BS-IB$_4$* (section 2.9.4.2) or 5% Giemsa staining (section 2.9.4.3). All other wells were cultured appropriately for at least 7 days, or until wells were confluent, after which they were stained with FITC conjugate *BS-IB$_4$* (section 2.9.4.2) for the evaluation of the α-gal epitope and 5% Giemsa (section 2.9.4.3) to give an estimation of surviving cell number.

2.10.2 Fluorescence Activated Cell Sorting (FACS)

FACS was evaluated as an alternative method for the selective removal of contaminating undifferentiated human ES cells. Before this could be achieved, the FACSAria was first assessed for its ability to successfully sort human ES cells, whilst maintaining their viability, pluripotentiality and sterility.

The FACSAria cell sorter (BD, UK) was equipped with an air-cooled argon ion laser emitting 15mW of blue light at 488nm and with standard filter setup. Detection of undifferentiated human ES cells used the parameter settings outlined in Table 2.10.2, using simultaneous measurements as previously described (Section 2.8.2). All cell analyses/sorting were performed using single colour staining, passed through a 70um nozzle using aseptically prepared PBS as sheath fluid and 70psi. At least 10,000 events were recorded for analysis, data were collected in list mode as pulse area signals (5 decades each on a logarithmic scale) and analysed using BD FACSDiva software.

2.10.2.1 Assessment of the viability of sorted human ES cells

Human ES cells were harvested into a single cell suspension as previously described (section 2.4), resuspended in 5mls of KO-DMEM. This suspension was left at room temperature for 10 minutes to allow any cell clumps to settle out. The supernatant from this was then collected by centrifugation at 260g for 5 minutes and resuspended in 0.2-1ml of culture medium containing 1x penicillin/streptomycin (100U/ml penicillin and 100µg/ml streptomycin). Data were acquired on a BD FACSAria by Dr Martin Waterfall (Roslin Institute, Edinburgh). The parameter settings for data acquisition were determined using unlabelled human ES cells and were maintained throughout all experiments (Table 2.10.2).

Detector	FSC	SSC	FL1	FL2
Voltage	250	225	335	300
Mode	Linear	Linear	Logarithmic	Logarithmic

Table 2.10.2: BD FACSAria acquisition parameters for human ES cells

Initially, cells were sorted according to their forward and side scatter profiles into 5ml polystyrene sterile tubes (Falcon), primed with 1ml CM, before being transferred back to cell culture plastic (NUNC) coated with an appropriate extracellular matrix, substituted with 1x penicillin/streptomycin (100U/ml penicillin and 100µg/ml streptomycin) for the first 24 hours. These cells were monitored for appropriate growth and morphology, normal karyotype (section 2.8.1) and continued pluripotentiality, including expression of ES specific cell surface markers (section 2.8.2), expression of alkaline phosphatase (section 2.8.3) and Oct-4 (section 2.9.4.1), differentiation into cell types representative of the 3 germ layers (section 2.8.4) and

directed differentiation into the osteogenic lineage (section 2.8.5), following 5 passages in culture.

2.10.2.2 Establishing the regulation kinetics of ES cell surface markers

H9 wild type and transgenic M2 ES cells were differentiated in an undirected manner, as monolayer cultures by the removal of conditioned medium and hbFGF and the addition of 10% FBS. The differentiation time course spanned a 3-week period, after which cells from a 75cm^2 tissue culture flask (for each time point) were harvested (section 2.4) and stained as previously described (section 2.8.2) for SSEA-4, TRA-1-81 and α-gal as markers of undifferentiated ES cell status, and SSEA-1 and GD2 as markers of differentiation. Data was acquired using the parameters previously determined (Table 2.10.2) for 0, 2, 4, 6, 9, 11, 13, 16 and 18 days in differentiation medium. This information was then used to determine the time point at which discrete cell populations could be isolated and subsequently sorted based on the presence or absence of a specific cell surface marker.

2.10.2.3 Sorting Positive and Negative cells from a mixed population

Cells from 6 and 8 days of differentiation were prepared (section 2.10.2.1) and stained (section 2.8.2) for sorting at previously described. Sorting was performed on M2 cells according to their expression of either α-gal or TRA-1-81, using the same defined parameters (Table 2.10.2), gated so that positively stained cells were directed right and unstained (negative) cells were directed left into 5ml polystyrene FACS tubes (Falcon). Sorted cells were then cultured along side unsorted cells

(differentiated and undifferentiated) in either ES cell culture (CM plus hbFGF) medium or differentiation cell culture (No hbFGF plus 10% FBS) medium at a cell density of 10^4 and 10^5 per well of a 12-welled plate (NUNC). Initially cell cultures were supplemented with 1x penicillin/streptomycin (100U/ml penicillin and 100µg/ml streptomycin) to reduce opportunistic bacterial infections, but this was removed in the first media change at 24 hours post-sort. Medium was routinely changed every 24hrs for ES and 48hrs for differentiation.

2.10.2.4 *Assessing the purity of sorted cells.*

Sorting purity was initially assessed through immunochemistry to determine the occurrence of false negative events. The negatively gated populations were stained as monolayers 48 hours post-sort with FITC conjugated *BS-IB4* or TRA-1-81 as previously described (section 2.9.4).

The persistence of contaminating ES cells was determined following 7-10 days in ES specific culture conditions. This assessment was based on cell morphology, Oct-4 expression (section 2.9.4.1), the presence of alkaline phosphatase (section 2.8.3), and the live/dead stain (Acridine Orange (AO)/ Ethidium Bromide (EtBr)).

Briefly, acridine orange (Sigma) and ethidium bromide (Sigma) 10mg/ml stock solutions were diluted in complete PBS (plus Mg^{2+}/Ca^{2+}) to give a working stock of 1mg/ml. Culture media was removed and the cells were washed twice with PBS plus. The AO/EtBr working stock was subsequently diluted 1:50 (0.2mg/ml) and used immediately. Cells were incubated with sufficient stain to cover the cells (0.5-

1ml), for 40 seconds, at room temperature in the dark. The stain was then removed and the cells washed twice with complete PBS, and finally resuspended in complete PBS after which the cells were photographed immediately. The stain and all washes were disposed of appropriately, filtered through charcoal to safely remove the ethidium bromide.

CHAPTER 3 DEVELOPMENT OF CELL CULTURE TECHNIQUES AND ESTABLISHMENT OF EXPERIMENTAL CELL LINES

3.1 Introduction

As discussed in section 1.8, human ES cells, first derived by James Thomson and colleagues in 1998 were isolated using a combination of mechanical and collagenase IV or dispase disaggregation agents and initially cultured on mouse embryonic fibroblast (MEF) feeder-cells, in the presence of serum and leukaemia inhibitory factor (LIF) (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998). Publication of the feeder-free culture for human ES cells in 2000, had significant implications for the scalability of human ES cell culture, growing cells on a synthetic extracellular matrix (matrigel) in medium pre-conditioned by MEFs and supplemented with recombinant human basic fibroblast growth factor (hbFGF) (Xu *et al.,* 2000). However, passage of human ES cells using the feeder-free technique continued to rely on dissociation

with collagenase IV, a consequence of which was that the ES cell colonies remained in small clumps at each passage (Xu *et al.,* 2000). Since 1999, the McWhir group has been investigating a method for the disaggregation of human ES cells using a trypsin/EGTA solution referred to as TEG (Thomson *et al.*, manuscript in preparation). Use of TEG promotes dissociation into single cells and resulted in monolayer cell growth, both of which were important properties for a number of the techniques used in this investigation.

The ability of human ES cells to grow indefinitely in culture has provided an opportunity to manipulate their fate, through genetic engineering. As discussed in Section 1.8.4, a number of different methods of transfection have been used for genetic manipulation of human ES cells, namely cationic lipids (lipofection) such as FuGene 6 (Park et al., 2003; Zwaka and Thomson 2003; Lebkowski *et al.,* 2001; Eiges et al 2001) and Lipofectamine 2000 (Hay et al., 2004; Eiges at al., 2001), polyethylenimine, ExGen 500 (Schuldiner et al., 2003; Zwaka and Thomson 2003; Eiges et al., 2001), viral transduction using both lentivirus (Martin et al., 2005; Gropp et al., 2003; Ma et al., 2003) and adeno-associated virus (AAV) (Smith-Arica et al., 2003) and more recently using electroporation (Zwaka and Thomson, 2003).

Electroporation, which has been widely used to engineer mouse ES cells (Kim *et al.,* 2002; Thomas & Capecchi 1987; Doetschman *et al.,* 1986, 1988), providing transfection efficiencies in the region of $2.1x10^{-5}$ to $5.9x10^{-3}$ (Lakshmipathy *et al.,* 2004; Piedrahita *et al.,* 1992), has proven problematic for human ES cells, due in part to high levels of cell death using standard mouse electroporation conditions. Zwaka

and Thomson (2003) reported that following optimisation of the standard mouse conditions the transfection efficiency of human ES cells was at best $2.33x10^{-5}$ using the BioRad Gene Pulse II system (Zwaka and Thomson, 2003). However, optimisation of electroporation for human ES cells, within the McWhir group (Priddle, H. unpublished data) using the Eppendorf Multiporator system and human ES cells dissociated with TEG, have shown a significant increase in the transfection efficiency of human ES cells in excess of 20-fold ($5.58x10^{-4}$).

The aim of this investigation is to engineer human ES cells so that they express cell surface markers specifically in their undifferentiated state. These markers will then be used to selectively eliminate undifferentiated human ES cells from differentiated populations using complement-mediated lysis or fluorescence activated cell sorting. Both of these processes, successful manipulation of the human ES cells and exposure of the transgenic cells to the elimination strategy, rely heavily upon processing human ES cells as a single cell suspension. This chapter will look at adapting the established *in vitro* culture conditions to improve growth of human ES cells as single cell cultures and at manipulating their genome to express cell surface epitopes of foreign origin.

3.1.1 Chapter Aims

1. To optimise the culture of established human ES cells lines, to support monolayer growth and disaggregation into single cell suspension.

2. To build expression vectors that would confer expression of cell surface markers under the control of ES cell specific promoters.

3. To use electroporation, established during the course of this investigation to integrate foreign genes into human ES cells.

4. To provide evidence of DNA integration, protein expression and functionality of the transgenes.

3.2 Results

3.2.1 The Adaptation of Existing Cell Culture Techniques for Established Human ES Cell Lines.

3.2.1.1 The Method of Disaggregation Impacts on How Human ES Cells Grow.

As previously mentioned, the expansion of human ES cells using the feeder-free technique relied on dissociation with collagenase IV (Xu *et al.,* 2000), which resulted in the ES cell colonies being maintained as small clumps at each passage. Furthermore, human ES cells cultured in this way were often associated with spontaneous differentiation into fibroblast-like cells (Figure 3.2.1a), and in sub-optimal conditions, the fibroblastic population would take over the culture, resulting in loss of the human ES cell component. Cell dissociation with EDTA is an alternative to collagenase IV. EDTA acts by chelating calcium and magnesium ions from the intercellular bridges and desmosomes, which results in cells then dissociating from each other and from the support matrix. However, like collagenase IV, disaggregation with EDTA resulted in small clumps of ES cells at passage and the presence of a fibroblast cell type within the culture (Figure 3.2.1b).

Trypsin/EGTA solution (TEG) is a method of disaggregation that has been piloted by the group since 1999, for passaging human ES cells (Thomson *et al.,* manuscript in preparation) as an alternative to both EDTA and collagenase IV dissociation. Trypsin is a pancreatic serine protease, which specifically acts on positively charged lysine and arginine side chains, leading to dissociation from extra cellular matrices. EGTA, like EDTA, is also a chelator of calcium ions, however its specificity for

calcium is greater than EDTA and is considered more efficient in breaking calcium dependent adhesion mediated by cadherins and selectins. Together, trypsin/EGTA (TEG) encouraged disaggregation into single cells and promoted monolayer cell growth instead of discrete colonies (Figure 3.2.1c), while significantly reducing levels of spontaneous differentiation to little if any at all (Figure 3.2.1c).

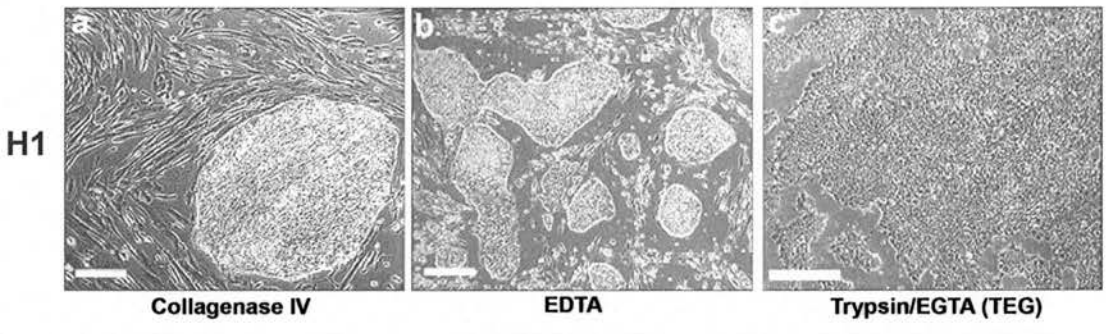


Figure 3.2.1: Disaggregation techniques for the passage of human ES cells (H1) impact on their growth pattern. Disaggregation with Collagenase IV (a) and 0.5mM EDTA (b) results in the formation of discrete colonies between which differentiated (fibroblast) cell types are observed. TEG disaggregation (c) on the other hand produces a monolayer of ES cells, with little if any evidence of differentiated cells types. Scale bars represent 100um

To determine the efficiency of the TEG system as a method for disaggregating human ES cells, the group compared it with collagenase IV and EDTA disaggregation, in terms of plating efficiency following passage, expression of cell surface markers and continued pluripotentiality for the established H9 cell line (Thomson *et al.,* manuscript in preparation). The results were very encouraging with an increased plating efficiency following TEG passage, continued pluripotentiality and expression of the characteristic cell surface markers (positive for SSEA-4, TRA-

1-60 and TRA-1-81 and negative for SSEA-1) (Thomson *et al.,* manuscript in preparation).

Consequently, the remaining cell lines (H1 and H7) available to the group were converted to the TEG passaging regime for use within this thesis. To substantiate the data for the H9 cell line, the plating efficiency of the H1 cell line was also assessed. The morphology of both H1 and H7 cell lines were monitored, as was the expression of characteristic cell surface marker following 10 passages.

3.2.1.2 Plating Efficiency

To determine the plating efficiency of human ES cells following disaggregation with either collagenase IV, EDTA or TEG, $1x10^5$ H1 human ES cells were plated in duplicate wells, of a 6-well plate, in triplicate. Twenty-four hours after plating, one well from each duplicate was harvested and counted. The second well was grown to confluence and then passaged appropriately, seeding duplicate wells at $1x10^5$ cells per well repeating the process. The plating efficiency of H1 human ES cells was monitored in this way for 10 passages using each of the disaggregation agents.

The results of the passaging regime experiment did not demonstrate an obvious advantage of one treatment over the others. Each of the passaging regimes produced high and low counts, with the exception of collagenase IV, which remained fairly constant with an average plating efficiency of 70.91% (+/- 5.99%: Figure 3.2.2). The average plating efficiency using EDTA disaggregation was almost identical although

highly variable (69.29% +/- 13.50%), TEG disaggregation was again very variable, but resulted in the highest plating efficiency, 82.15% (+/- 13.92%: Figure 3.2.2).

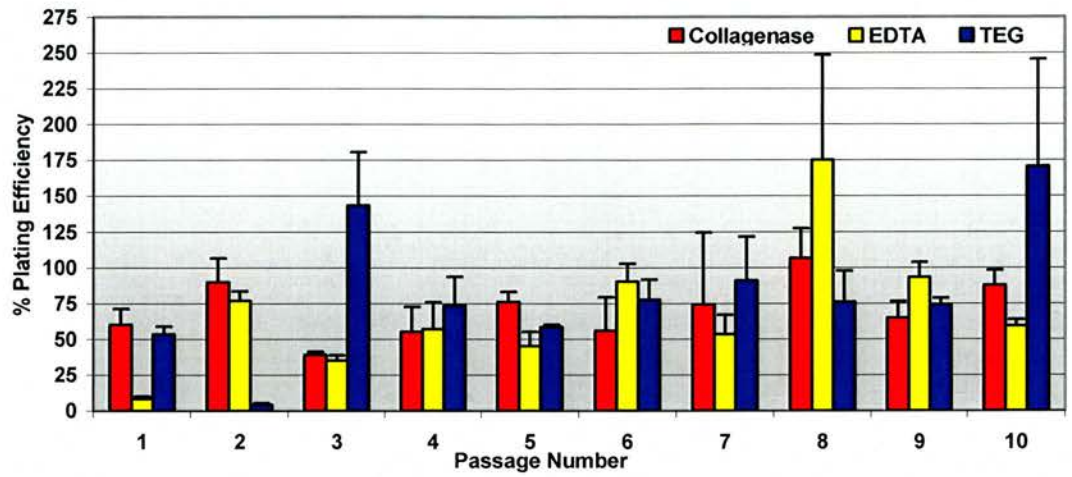


Figure 3.2.2: The estimated plating efficiency of the established human ES cell line, H1, using collagenase IV, EDTA and TEG as methods of disaggregation. Triplicate wells for each dissociation agent were counted 24 hours after re-plating using a standard haemocytometer, and the mean used as an estimate of the plating efficiency of that agent. Error bars represent the standard error of the mean.

The estimation of plating efficiencies for collagenase IV and EDTA were heavily over estimated, since for both passaging regimes the cell counts could not distinguish between undifferentiated human ES cells and the differentiated human ES cells or fibroblasts (Figure 3.2.1). Since the use of TEG, as a disaggregation agent, was not associated with cellular differentiation, it appeared that these data agreed with those obtained for the H9 cell line (data not shown); the plating efficiency of H1 cells was increased following continued disaggregation with TEG (Figure 3.2.2). A similar trend was also observed for the H7 human ES cell line dissociated using TEG instead

of collagenase IV, however, the plating efficiency was not calculated for this cell line.

3.2.1.3 Maintenance of ES Cell Characteristics

Expression of the cell surface markers, SSEA-4, SSEA-1, TRA-1-60 and TRA-1-81 has been used extensively to identify undifferentiated human ES cells (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998). Figure 3.2.3, shows analysis by flow cytometry of this panel of cell surface markers for undifferentiated H7 cells cultured following collagenase IV dissociation (Figure 3.2.3a-e) or TEG dissociation (Figure 3.2.3f-j). These data clearly show a reduced level of differentiation within the population disaggregated using TEG, only 15% of the cells expressed SSEA-1, a marker associated with differentiation, compared to 63% when collagenase IV disaggregation was used. Furthermore, in every other case, the histogram profiles for TEG passaged cells show more discrete populations with much cleaner distinction between those which were positive and the isotype controls, indicating a more uniform population. This difference in the expression of the cell surface markers was also observed for both the H1 and the H9 cell lines when converted to passage using TEG dissociation instead of collagenase IV (data not shown).

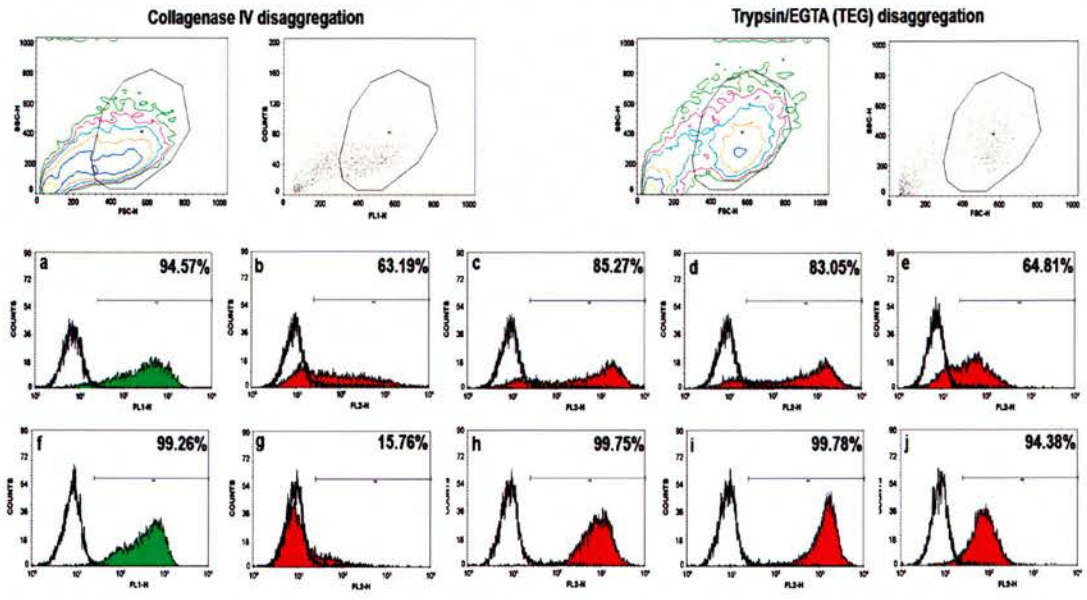


Figure 3.2.3: Expression of characteristic cell surface markers of human ES cells change slightly using TEG disaggregation instead of collagenase IV as a result of removing the differentiated population the histogram peaks are much better defined and the level of the differentiated marker (SSEA-1) markedly reduced. Images a-e represent H7 cells disaggregated with collagenase IV, while images f-j, show the same population transitioned to disaggregation with TEG showing cell surface expression of SSEA-4 (a&f), SSEA-1 (b&g), TRA-1-60 (c&h), TRA-1-81 (d&I) and CD9 (e&j). Percentages represent the proportion of positive cells within the gated (live) cell population.

Along with the expression of characteristic cell surface markers, human ES cells dissociated with TEG also expressed the transcription factor Oct-4 and alkaline phosphatase (Figure 3.2.4). Oct-4 staining is nuclear and in undifferentiated human ES cell cultures it was shown to co-localise with the nuclear specific stain, Dapi. All nuclei in the TEG passaged cultures which were positive for Dapi, were also shown to express Oct-4, confirming the absence of the differentiated ES or fibroblast cell types observed in collagenase IV disaggregated ES cells (Figure 3.2.4a and b).

Likewise all cells with ES cell morphology were positive for alkaline phosphatase (Figure 3.2.4c)

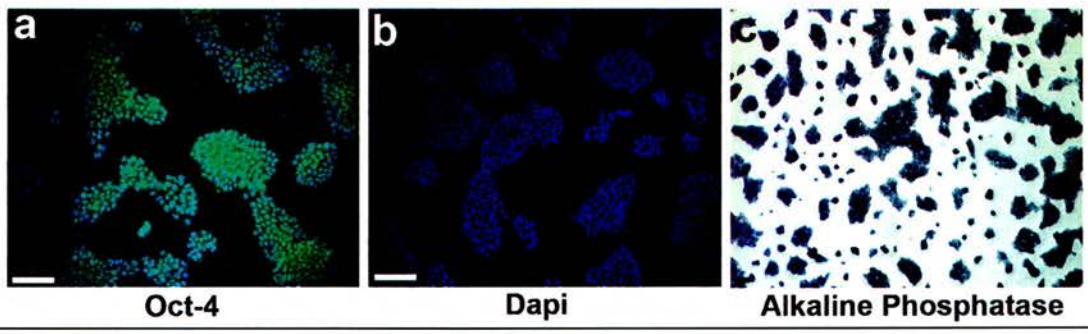


Figure 3.2.4: Undifferentiated human ES cells cultured using TEG dissociation, express Oct-4 (a) and alkaline phosphatase (c). The whole of the TEG culture express Oct-4 with no evidence of nuclei from differentiated cells as indicated in panel b, which shows non-specific nuclear staining with dapi. All scale bars represent 100um.

3.2.1.4 Human ES Cells in Long-Term Culture using TEG Dissociation Possess a Stable Karyotype.

During the initial passage regime, culture of H9 cells using TEG as a method of disaggregation resulted in the occurrence of a specific chromosomal abnormality, a complete duplication of the short arm of chromosome 7 and loss of the long arm (an isochromosome 7p) (Thomson *et al.,* manuscript in preparation). Since this observation, a number of other laboratories have described the occurrence of karyotypic abnormalities following different types of enzymatic disaggregation, including collagenase IV (Thomson *et al.,* in preparation; Mitalipova *et al.,* 2005; Brimble *et al.,* 2004; Inzunza *et al.,* 2004; Rosler *et al.,* 2004; Draper *et al.,* 2004). To determine if this abnormality was a random event or a consequence of TEG

disaggregation, H1, H7 and H9 cells were assessed for their continued karyotypic stability following prolonged TEG disaggregation.

It proved difficult to obtain sufficient mitotic spreads to perform karyotype analysis on both the H1 and H7 cell lines (and due to the time constraints of this thesis it was decided that time would be better spent on engineering the cells first and then determining their karyotype if time permitted). However, mitotic spreads were successfully prepared from derivatives of the H9 cell line, the clonal cell line M2 (see section 3.2.2.2) following 10 and 49 passages with TEG, post transfection at passage 56.

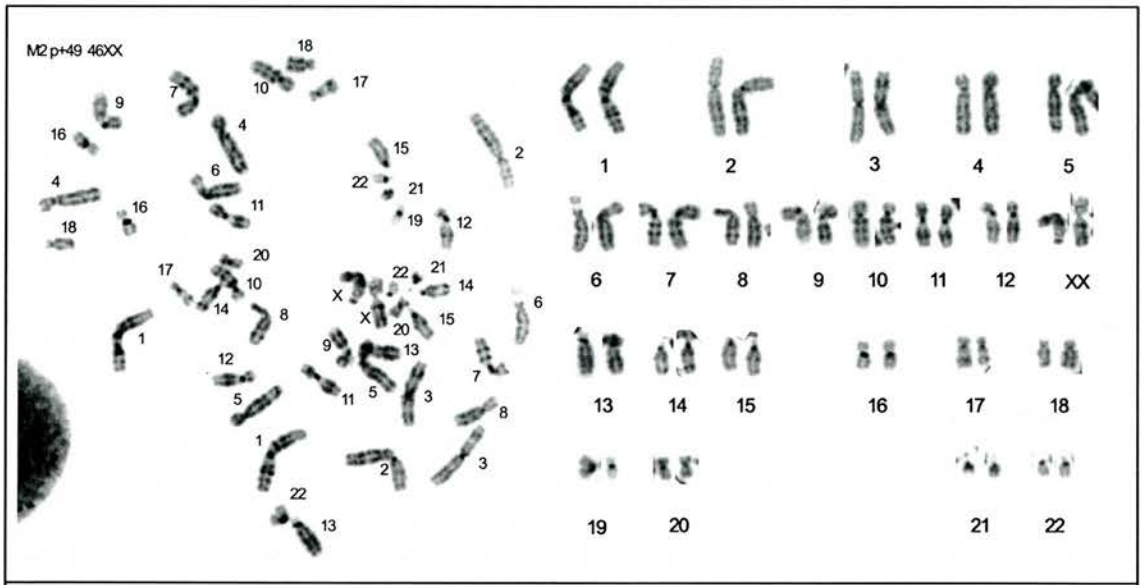


Figure 3.2.5: A representative example of a mitotic spread from the clonal cell line M2 following 49 passages post-transfection at passage 56. Karyotype profile is 46XX normal, with no obvious chromosomal abnormalities. Images produced in collaboration with J. Fletcher Roslin Institute.

A total of 30 spreads were counted, 15 of which were fully analysed (Fletcher, J. Roslin Institute) showing a stable 46 XX karyotype (Figure 3.2.5). These data suggested that the initial finding of the abnormal chromosome 7 was probably not a result of the enzymatic dissociation, but more likely to be that the cells had at some point been stressed, placing them under selective pressure, where survival was dependent upon the presence of the isochromosome 7p. Since the report of chromosomal abnormalities by Draper *et al.* (2004) a number of reports have been published that contradict the speculation that human ES cells have unstable karyotypes over time in culture (Sjogren-Jansson *et al.,* 2005; reviewed by Hoffan & Carpenter 2005). One might suggest that perhaps the culture conditions currently employed for the propagation of human ES cells are sub-optimal and that further refinement might address the occurrence of chromosomal abnormalities.

3.2.2 Engineering Human ES Cells to Express Lysis Epitopes on Their Cell Surface.

The ability of human ES cells to grow indefinitely in culture provides an opportunity to manipulate their fate, through genetic engineering. The selective ablation strategies in this thesis rely on achieving cell surface expression of foreign "lysis" or "selection" epitopes.

3.2.2.1 H2-K^k - A Murine Major Histocompatibility Complex Antigen Genetic Manipulation

To determine the potential of the murine major Histocompatibility complex (MHC) antigen, H2-K^k, to initiate a complement mediated attack, a preliminary experiment to engineer cell surface expression on the human ES cell line H7 was attempted. Initially there were two H2-K^k transfection strategies, one based on the use of the Oct-4 promoter and the other based on the hTERT promoter. A schematic representation of the two expression vectors can be seen in Figure 3.2.6 while specific details of the cloning strategy can be found in Appendix I.1.

In the first instance phTH2-KkpAPN was stably transfected into 10^6 wild type H7 human ES cells by electroporation (Multiporator, Eppendorf). Following electroporation, half of the cells were plated under 200μg/ml G418 selection, a concentration previously determined by Dr A Thomson (Roslin Institute, Edinburgh) as being optimal for the removal of non-resistant human ES cells, in a time period suitable for deriving clones, 7 to 10 days. The resistant colonies (>300) were

subsequently pooled and the resulting cell line referred to as H7-K^k. The rationale for pooling the colonies for this preliminary experiment was to balance the effects of site-specific gene integration to provide a global picture of H2-K^k expression, thereby reducing the volume of analysis in the first instance.

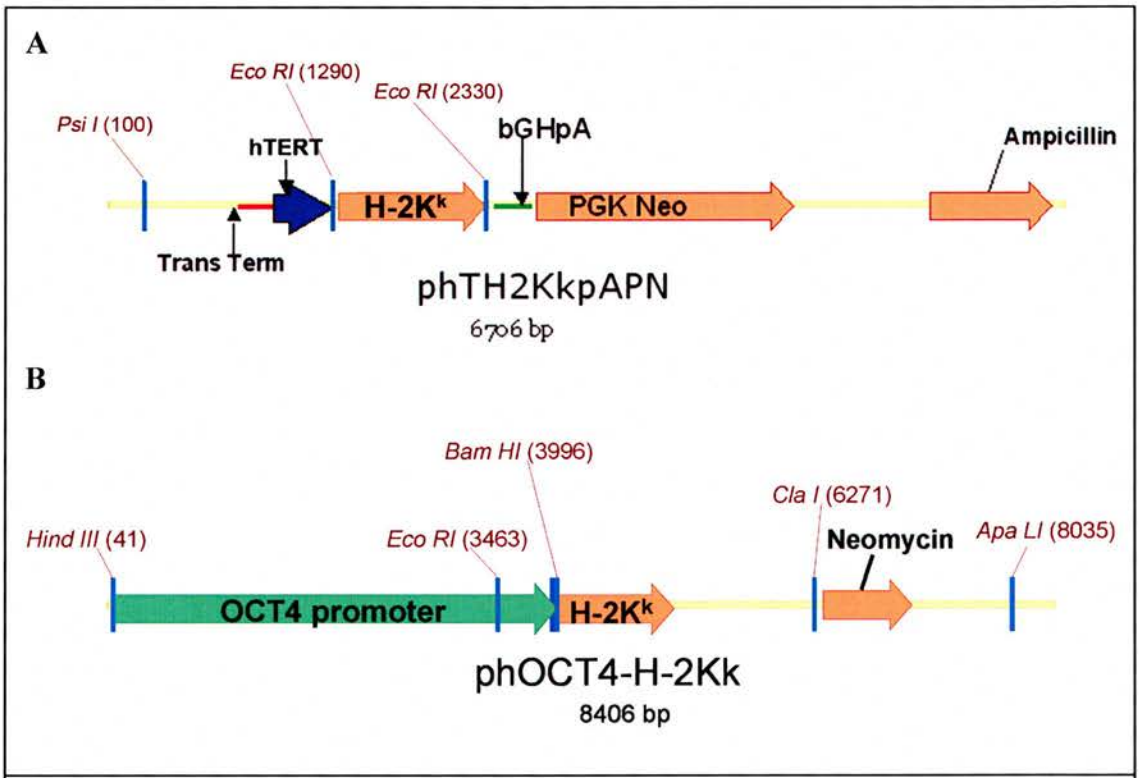


Figure 3.2.6: A schematic representation of the H2-K^k expression constructs. A] H2-K^k was cloned by PCR from *pMAC.KkII* (Milteny Biotech) and TA cloned into pGEM TEasy (Promega) before being subcloned as an Eco R1 fragment into phTpAPN (Priddle H). *PsiI* was used as a site for linearising the construct. B] using an *AgeI* : *HpaI* digest the eGFP minigene was removed from phOCT-4-eGFP (Zhao D) and the H2-K^k PCR product subcloned in as a blunted *EcoR1* fragment. *Apa LI* was used as a linearisation site.

Analysis of H2-K^k Protein Expression

Immunochemistry, using commercially available monoclonal anti-mouse H2-K^k antibody and adult CBA mouse lung-fibroblasts, was used to optimise staining before assessing expression of H2-K^k on the transgenic cell line. Wild type H7 cells were used as a negative control. Optimal conditions for H2-K^k immunochemistry were determined following several attempts with different methods of fixation, and antibodies from different suppliers using a range of dilutions (Table 3.2.1).

Fixative	Primary H2-K^k antibody dilution			Secondary Antibody dilution	
	BD PharMingen (0.5mg/ml)	**FITC-Miltenyi Biotech (12µg/ml)**	**FITC-Southern Biotech (0.5mg/ml)**	**IgG-FITC (1.0mg/ml, Sigma)**	**FAB-Rhodamine (1.5mg/ml, Jacksons)**
70% Methanol	1:10 1:25 1:50 1:100	1:10 1:25 1:50 1:100		1:100 1:200 1:500	1:100 1:250 1:500
2% PPFA	1:10 1:50 1:100	1:10 1:50 1:100			1:250
100% Ice cold Acetone	1:25 1:50 1:100 1:200			1:100 1:200 1:500	
Antibody staining prior to fixation with 4% PFA	1:25 1:50 **1:100** 1:200	1:25 1:50 1:100 1:200	**1:100**	1:100 **1:200** 1:500	
(1:1) 50% Methanol: 50% acetone Ice cold	1:50			1:200	
Absolute Ethanol	1:100		1:100	1:200	

Table 3.2.1: Conditions used in the optimisation of H2-K^k immunochemistry showing a range of fixatives, and concentrations of different primary and secondary antibodies. The conditions in bold type were determined to be optimal. FITC - Fluorescein Isothiocyanate, PFA - Paraformaldehyde

The most convincing cell surface staining was achieved when cells were stained before fixation using primary antibody diluted 1:100 (BD Pharmingen) with a fluorescein isothiocyanate (FITC) conjugated secondary antibody (Sigma) diluted 1:200. However, staining using the same antibodies and dilutions was also observed when cells were permeablised and fixed for 2 minutes in absolute ethanol, although the pattern of expression altered slightly, showing more internal staining than cell surface (Figure 3.2.7 a and b).

To determine that the staining for H2-K^k was specific, the expression pattern of β-2 microglobulin (β-2M), an MHC class I associated protein, was also performed by immunochemistry using a commercially available monoclonal goat anti-β-2M antibody, which cross reacts with both human and mouse β-2M (Santa Cruz Biotechnologies, UK) with a R-phycoerythrin (R-PE) conjugated secondary antibody (Autogen Bioclear, UK) (Table 3.2.2).

Fixative	Primary β-2M antibody dilution Santa Cruz Biotechnologies, UK	Secondary Antibody dilution Autogen Bioclear, UK
Absolute Ethanol	0.2mg/ml diluted 1/100	IgG2b-R-PE 0.4mg/ml diluted 1/200
Live staining post –fixed with 4% PFA	**0.2mg/ml diluted 1/100**	**IgG2b-R-PE 0.4mg/ml diluted 1/200**

Table 3.2.2: Conditions used in the optimisation of β-2 microglobulin (β-2M) immunochemistry showing a range of fixatives, and concentrations of different primary and secondary antibodies. The conditions in bold type were determined to be optimal. R-PE - R-phycoerythrin.

An identical staining pattern was observed for expression of β-2M in both live and ethanol permeabilsed adult CBA lung fibroblasts to that previously observed for H2-

K^k (Figure 3.2.7 d and e). Furthermore, double staining for β-2M and H2-K^k (using a FITC conjugated H2-K^k antibody, Southern Biotech, USA) showed that these two proteins co-localised both with live and ethanol fixed cells (Figure 3.2.7 g and h).

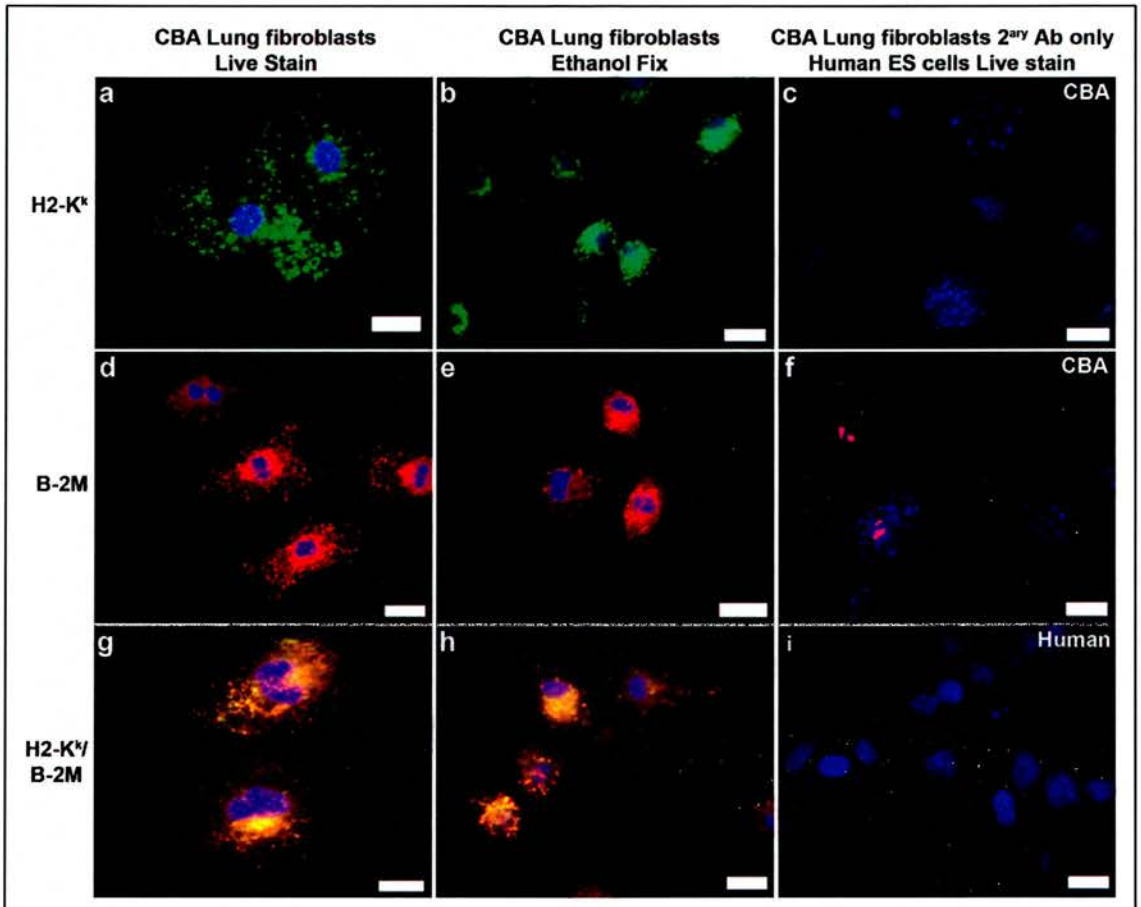


Figure 3.2.7: Immunochemistry for H2-K^k and β-2 microglobulin (β-2M) shows positive expression in CBA adult fibroblasts, but not in transgenic human ES cell lines. Cell surface expression of both H2-K^k (a and b FITC) and β-2M (d and e R-PE) were observed when CBA lung fibroblasts were stained live (a, d and g) or permeablised with absolute ethanol (b, e and h). Double staining with H2-K^k (FITC) and β-2M (R-PE) showed co-localised expression of these two genes (g and h). However, transgenic H7-K^k-ES cells were negative for both H2-K^k and endogenous β-2M (i). Images c and f show that H2-K^k and β-2M staining are specific to the presence of the respective primary antibody. Scale bars represent 100um.

However, when the transgenic H7-K^k cell line was assessed for its level of cell surface H2-K^k expression, no specific staining was observed under any of the conditions tried (Figure 3.2.7i is a representative image). Furthermore, positive cell surface staining for the endogenous MHC class I associated protein (β-2M) was also absent (data not shown). Lack of cell surface β-2M suggested that human ES cells either lack MHC expression or express at levels below those detectable by immunochemistry, a hypothesis that was confirmed by Drukker et al (2002).

Integration of H2-K^k cDNA was confirmed by RT-PCR.

To determine if the integrity of the H2-K^k transgene had been compromised during subcloning, RT-PCR was performed to confirm successful subcloning and integration of the H2-K^k gene into the H7 human ES cell genome.

Mouse and human specific trans-intronic primers had been designed to detect β-Actin mRNA (a kind gift from Dr S. Pells and Dr N. Forsyth, Roslin Institute respectively) and were used to generate loading controls (Figure 3.2.8 lanes 1 and 3-4 respectively). Lane 2 represents RT-PCR in the absence of reverse transcriptase indicating the specificity of PCR to cDNA and not genomic DNA contamination. RT-PCR for the presence of H2-K^k mRNA showed positive expression in both the adult CBA lung fibroblasts (lane 5) and a weaker band in the transgenic H7-K^k cell line (lane 7), but an absence of expression in the H7 parental population (lane 6) as expected (Figure 3.2.8). These RT-PCR data show that integration and transcription of the H2-K^k transgene had successfully been achieved in the H7-K^k cell line,

suggesting that there may be an issue with appropriate translation and transportation of the protein.

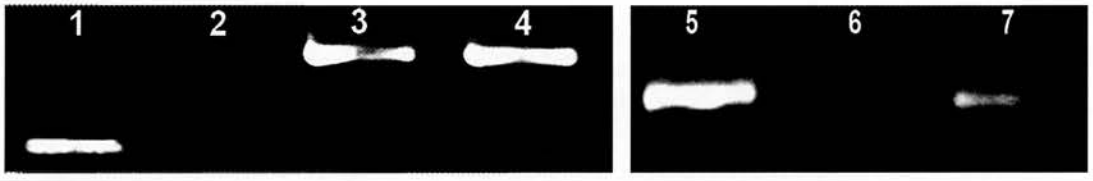


Figure 3.2.8: RT-PCR for β-Actin (1-4) and H2-K^k (5-7). CBA adult lung fibroblasts RNA (1), wild type H7 RNA (3) and H7-K^k cell line (4) positively expressed species-specific β-Actin. Lane 2 represents a minus reverse transcriptase control. Positive H2-K^k expression was observed in both the CBA positive control RNA (5) and the H7-K^k transgenic cell line (7), but was not expressed in the H7 parental cell line (6).

Several unsuccessful attempts to determine the expression of the H2-K^k protein by Western Blot were made. Simultaneously, the expression of β-actin was assessed, using the same protein lysates as for the H2-K^k western blot, with success (Figure 3.2.9 A). The presence of β-actin indicated intact protein in the protein lysates in sufficient quantity for detection by western blot. Furthermore, it provided evidence that the solutions and technique were not responsible for the lack of H2-K^k expression, leaving only the H2-K^k antibody and conditions.

H2-K^k antibody, from different suppliers, was tried using a range of dilutions, in reducing and non-reducing conditions without success (Table 3.2.3). Under reducing conditions, plus DTT in the loading buffer, H2-K^k western blots were blank (data not shown) even with high concentrations (1:25) of antibody. It was hypothesised that since immunochemistry of H2-K^k was dependent upon the method of fixation, that

perhaps under reducing conditions the antibody was no longer capable of detecting its epitope, thus non-reducing conditions, minus DTT, were tried.

Antibody Supplier	Dilutions	Reducing conditions	Non-reducing conditions
BD Pharmingen (0.5mg/ml)	1:500, 1:200 and 1:100	XXX	
Southern Biotech (0.5mg/ml)	1:100, 1:50 and 1:25	XX	XXX

Table 3.2.3: Primary antibody conditions used for western blot analysis of the H2-K^k protein. Non-reducing conditions could not be determined using the H2-K^k antibody supplied by BD Pharmingen due to discontinuation of this product.

Under non-reducing conditions, faint bands of approximately the expected 41kDa size, were present in the positive control. Increased antibody concentration (1:25) strengthened these bands, but also resulted in a strong non-specific band in all of the cell samples, including the negative control, of the same size (Figure 3.2.9 B lane 2).

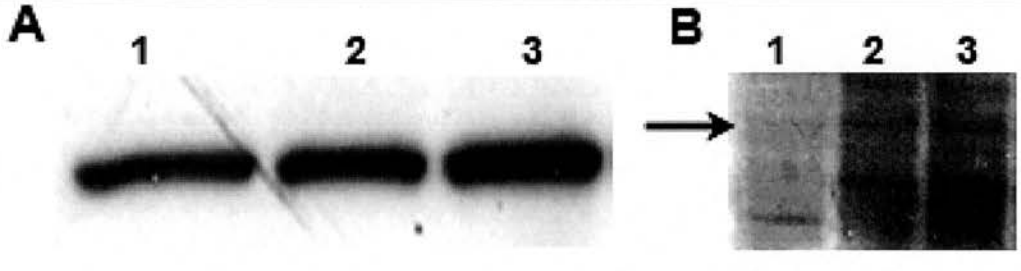


Figure 3.2.9: Protein expression analysis by western blot for β-Actin (A) and H2-K^k (B). Protein extracted from lysates of in vitro cell cultures of adult CBA lung fibroblasts (1), H7-ES cells (2) and transgenic H7-K^k cells (3). The arrow indicates the expected 41kDa H2-K^k band.

Since the data sheets for the commercially available H2-K^k antibodies did not indicate successful use in western blot analysis, it was decided that thesis time-constraints meant optimisation of this technique was inadvisable.

Co-expression of exogenous β-2M was insufficient to induce cell surface expression of H2-K^k

The cell surface expression of MHC molecules requires association with β-2M prior to transportation from the endoplasmic reticulum to the cell surface (Paulsson *et al.,* 2001; Williams *et al.,* 1989). Human ES cells are reported to have low levels of both MHC and β-2M expression (Drukker *et al.,* 2002). It was therefore hypothesised that a potential explanation for the lack of H2-K^k at the cell surface was a result of either insufficient β-2M to associate with both endogenous and transgenic MHC or perhaps a consequence of species specificity to β-2M association.

In an attempt to overcome this issue, a pooled cell line, co-expressing the H2-K^k construct and a constitutive mouse β-2M construct (Vcβ2 – Morello *et al.,* 1982) (kindly gifted by Prof. S. Ono, University College London, UK) was generated by electroporation (H7-K^k/β2) and stained for H2-K^k and β-2M expression. Unfortunately, the co-transfection of H2-K^k and β-2M in human ES cells had no impact on the level of cell surface expression of H2-K^k (Figure 3.2.10).

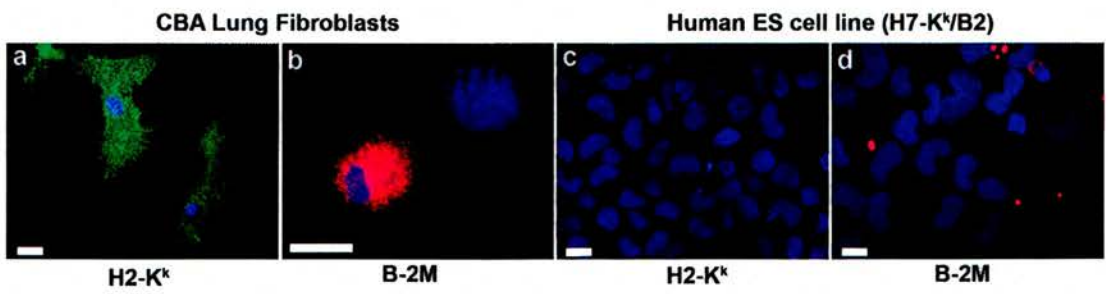


Figure 3.2.10: The co-transfection of β-2M and H2-K^k was insufficient to encourage cell surface expression of H2-K^k in H7-K^k/β2. Positive staining was for H2-K^k was observed in CBA lung fibroblasts (a) but not on H7-K^k/β2 ES cells (c). Likewise β-2M positive staining was also observed on CBA lung fibroblasts (b) but not H7-K^k/β2 ES cells (d)

An attempt was made to induce cell surface presentation of both MHC class I and β-2M by culturing the H7-K^k and -K^k/β2 cells in the presence of interferon-gamma (IFN-γ), a known stimulant of MHC class I (Rosa *et al.,* 1984; King & Jones 1983). However, initial attempts failed to show any improvement and since there was no evidence to suggest that the H2-K^k protein was present, it was decided that there was no justification in pursing this line of investigation.

The lack of cell surface H2-K^k expression and the potential requirement for the presence of additional genes and incubation with IFN-γ, suggested that this epitope was not suitable for its desired purpose, as a therapeutically useful lysis epitope. Therefore, the decision was taken to focus on using the α1,3galactosyltransferase gene and α-gal epitope as an alternative.

3.2.2.2 Galα1-3Galβ1-4GlcNAc-R (α-gal) Epitope

Genetic Manipulation

The α1,3galactosyltransferase (α1,3Gal) expression vectors used in this study were constructed by Dr. Helen Priddle (University of Nottingham, UK). A schematic representation of these can be seen below (Figure 3.2.11) with specific cloning details described in Appendix I.2.

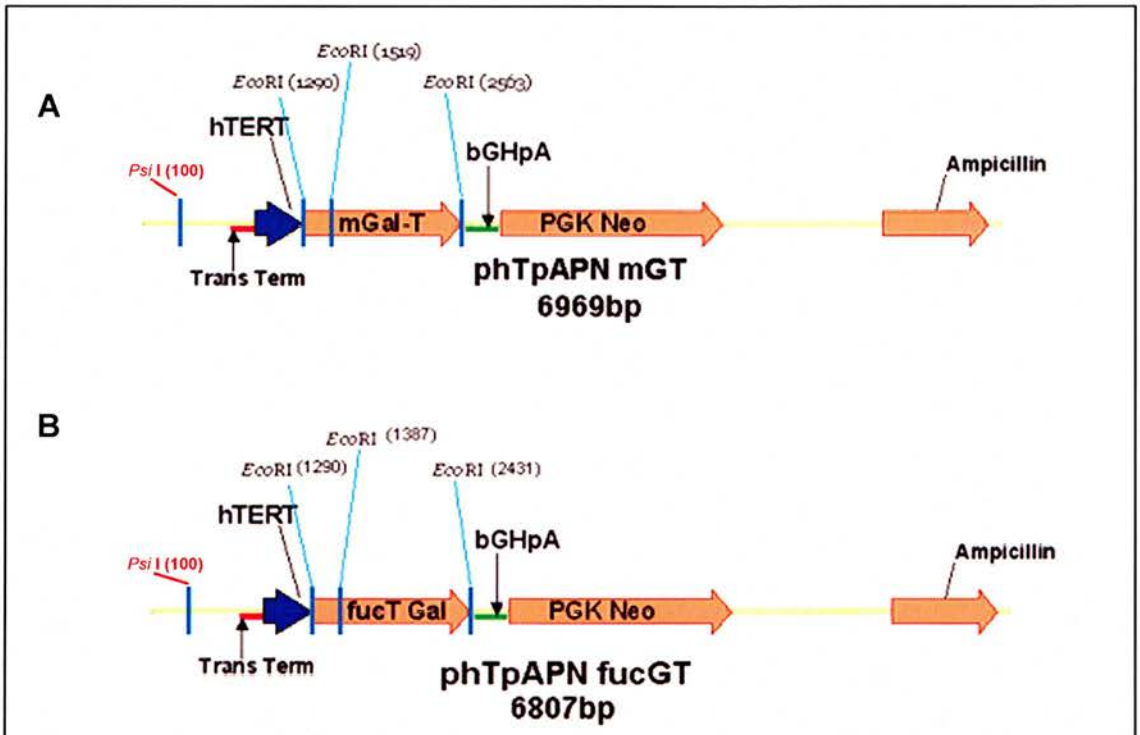


Figure 3.2.11: Schematic representation of the two constructs used to express the α-gal epitope on the cell surface of human ES cells. Both constructs were engineered by Dr Helen Priddle, a] mouse kidney α1,3galactosyltransferase cDNA under the control of a core hTERT promoter b] the same construct containing the leader sequence of the fucosyltransferase gene.

Dr H. Priddle produced transgenic mGT and fucGT cell lines, by electroporation using hyposmolarity buffer and an Eppendorf Multiporator (Eppendorf). Briefly,

$1x10^6$ H9 human ES cells were electroporated with linear DNA, plated and selected in the presence of 200μg/ml G418 for 10-14 days. From each transfection, yielding 558 and 585 colonies respectively, 24 colonies were picked and expanded, from which 4 of the mGT clones and 8 of the fucGT clones expressed the α-gal epitope. The latter clones contained both the α1,3Gal gene and the leader sequence of the fucosyltransferase gene (fucGT), which was developed because of the report that native α1,3Gal was out competed by fucosyltransferase for location in the Golgi apparatus, conferring preference to fucosyltransferase processing (Osman *et al.*, 1996). Thus by engineering the α1,3Gal construct to contain the leader sequence of the fucosyltransferase gene the hypothesis was that α1,3Gal expression would be elevated due to increased competition for location in the Golgi.

Analysis of Protein Expression

Expression of the α-gal epitope was determined using immunochemistry and flow cytometry using the α-gal specific fluorescein isothiocyanate (FITC)-conjugated *Bandeiraea simplicifolia* isolectin B4 (*BS-IB$_4$*, Sigma) described by Wu *et al.*, (1995). Based on unpublished data one mGT clone, M2, (Priddle H., unpublished data) was selected for use in this investigation, due to its known high level of expression. When cultured under G418 selection (200μg/ml), this cell line expressed the α-gal epitope on 95.40% of its cell surfaces at levels slightly lower than those of the endogenously expressing ovine (Polled Dorset) foetal fibroblast (PDFF) cell line (with a median fluorescence intensity of 478.29 and 615.27 respectively) (Figure 3.2.12A, images a and c).

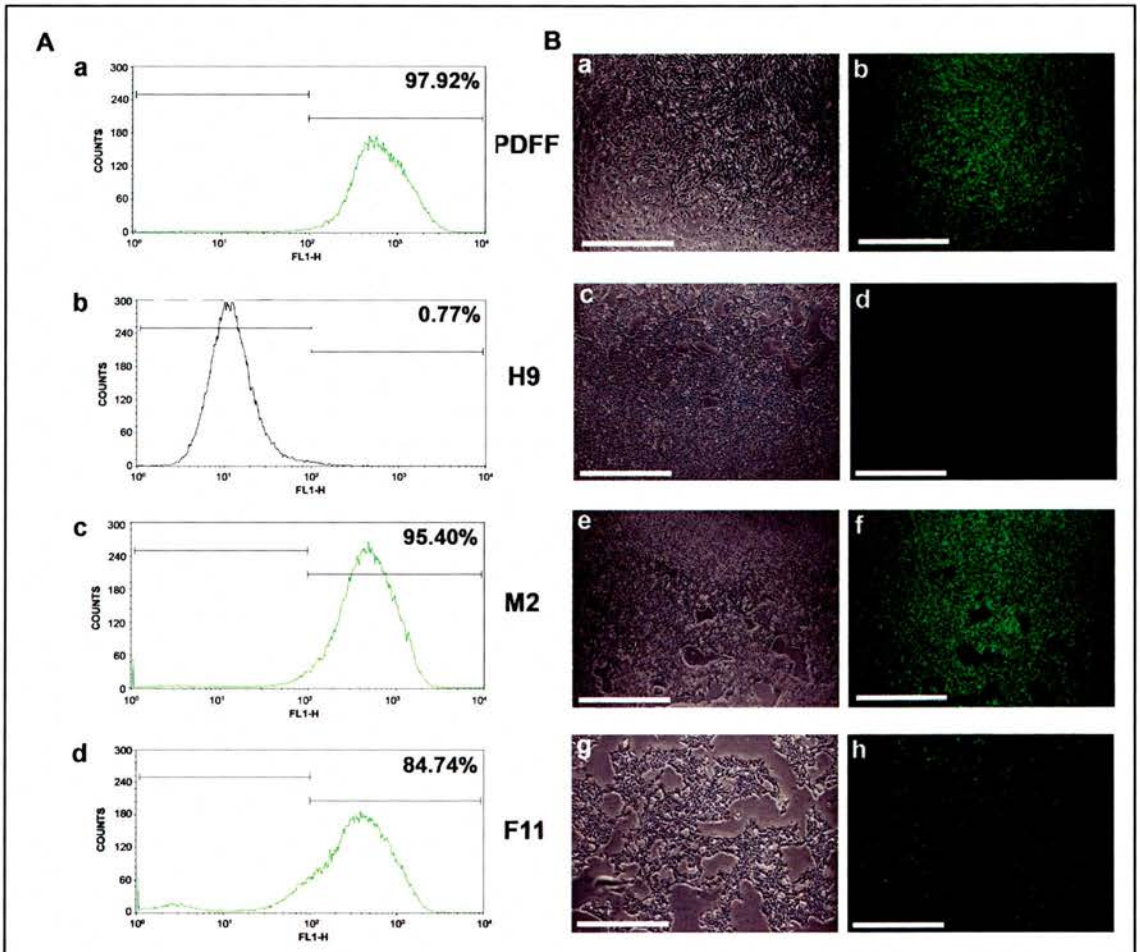


Figure 3.2.12: Expression of α1,3Gal was assessed using a fluorescein isothiocyanate (FITC)-conjugated Bandeiraea simplicifolia isolectin B4 (*BS-IB$_4$*, Sigma) specific for α-gal. A] Flow cytometry analysis of *BS-IB$_4$* staining on endogenously expressing PDFF (a), negative wild type human ES cells H9 (b) and transgenic cell lines M2 (c) and F11 (d). B] *BS-IB$_4$* immunochemistry for α-gal, on PDFF (a-b), H9 (c-d), M2 (e-f) and F11 (g-h) showing phase contrast and FITC images. Scale bar represents 100um

As a comparison the level of α-gal expression of a second transgenic clone (F11), this time containing the leader sequence of the fucosyltransferase gene, was assessed. The F11 cell line expressed lower levels of the α-gal epitope than did the M2 clone, with a median fluorescence intensity of 395.96 and appeared to be more variegated

with only 84.74% of the cells expressing (Figure 3.2.12A, image d), suggesting that the inclusion of the leader sequence of the fucosyltransferase gene had not increased the cells ability to process α1,3Gal in the Golgi.

Figure 3.2.12B shows visually the pattern of FITC conjugated *BS-IB4* binding for M2 (e-f) and F11 (g-h) clones in addition to PDFF cells as a positive control (a-b) and consistent with the inactive gene in humans, H9 wild-type cells were negative for α-gal (c-d). The immunochemistry and flow cytometry data both agreed that the M2 cell line had a higher and more uniform expression pattern than the F11 cell line, although there remained a degree of variegation within the M2 population. Based on these data, M2 was the cell line of choice in subsequent experiments, maintained in 200μg/ml G418 selection to help minimize the impact of position effect variegation (PEV).

Transgenic human ES cells carrying the α1,3Gal gene maintain a stable karyotype and pluripotentiality.

To ensure that genetic manipulation had not been detrimental towards the ES cells in terms of their pluripotentiality, embryoid bodies (EB's) were generated from the M2 cell line (see section 2.8.4). Following 7 days in suspension, the EB's were plated onto gelatin and allowed to spontaneously differentiate for 14 days in basic differentiation medium (KO-DMEM supplemented with 10% (v/v) FBS, 0.1mM NEAA, 2mM L-glutamine and 0.1mM β-mercaptoethanol). The resulting cells were then analysed for markers representative of all 3 embryonic germ layers, endoderm

(α-fetoprotein; AFP), ectoderm (β-tubulin III) and mesoderm (muscle specific-actin) (Figure 3.2.13 a-c respectively).

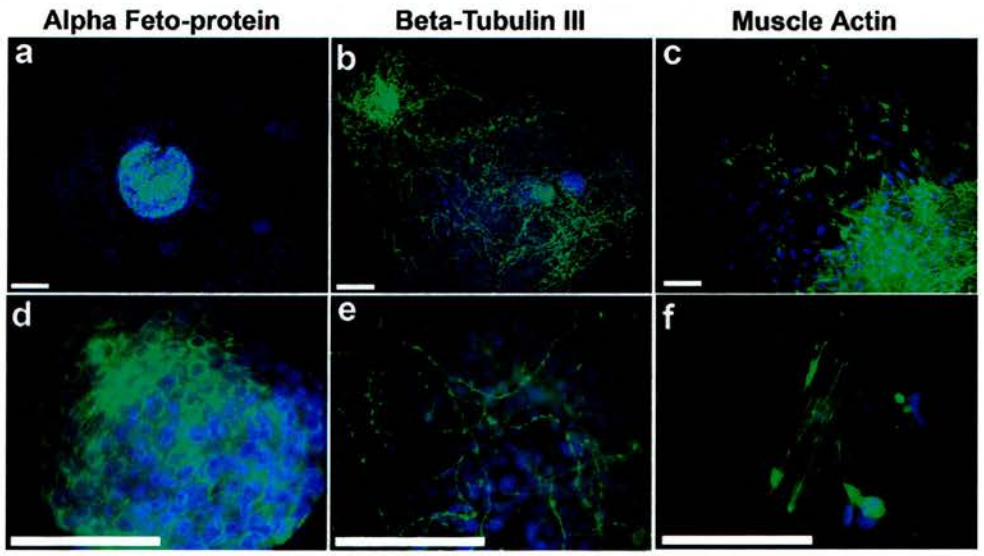


Figure 3.2.13: Transgenic M2 cells maintain their ability to differentiate in vitro into all 3 embryonic germ layers, endoderm, ectoderm and mesoderm. In vitro differentiation of M2 cells results in cell types, which express markers of endoderm (α-fetoprotein; AFP (a and d)), ectoderm (β-tubulin III (b and e)) and mesoderm (muscle specific actin (c and f)). All scale bars represent 100um.

Furthermore, when undifferentiated M2 cells were injected intramuscularly into severe combined immune deficient (SCID) mice they formed tumours. Upon analysis, these tumours were found to contain cell types representative of all 3 germ layers and were consequently classified as teratomas (Figure 3.2.14). For human ES cells, these are the only methods available to determine the pluripotentiality of the cells.

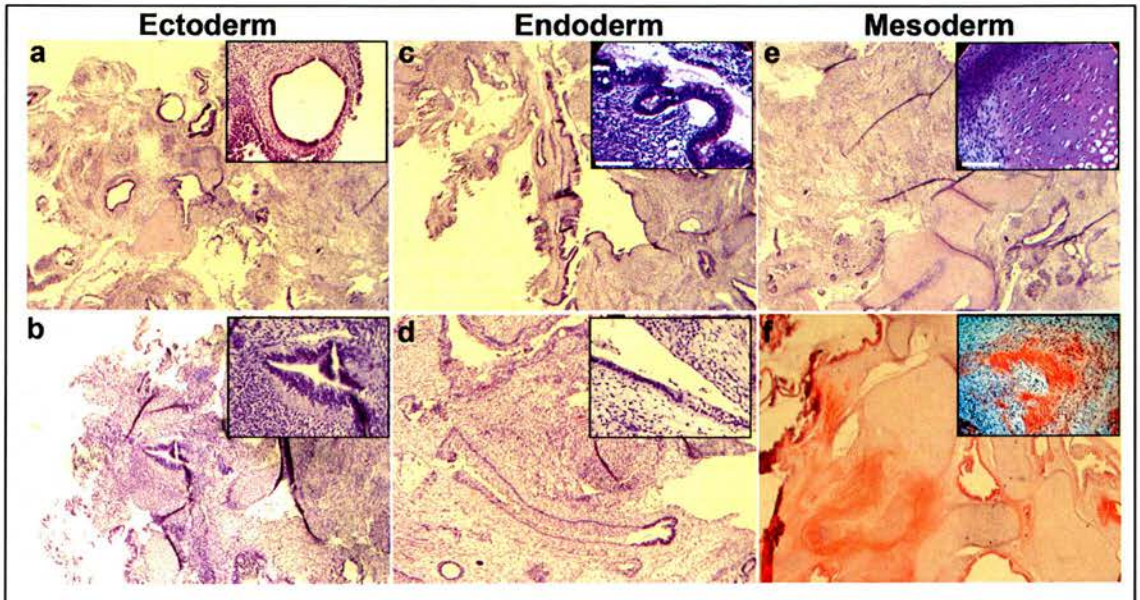


Figure 3.2.14: Transgenic M2 cells maintain their ability to differentiate *in vivo* into all 3 embryonic germ layers, endoderm, ectoderm and mesoderm. When injected intramuscularly into SCID mice M2 cells formed teratomas that contained a) sensory epithelium and b) neural epithelium from the ectoderm lineage, c) secretary epithelium and d) transitional ciliated epithelium from the endoderm lineage and e) cartilage, mesenchyme and f) smooth muscle from the mesoderm. Images provided by Dr David Brownstein (University of Edinburgh, Scotland) main images were taken using a x20 objective (inserts used a x100 (except b which used a x200) objective). Where provided scale bars represent 100μM

As previously mentioned in section 3.2.1.4, M2 cells maintained a stable karyotype after more than 60 passages with TEG. Together these data suggested that the genetic manipulation had not had a detrimental impact on the normality or pluripotentiality of the human ES cells.

Transgenic α-gal epitopes successfully bind human anti-α-gal immunoglobulins.

To elicit complement-mediated lysis, it is essential that the transgenic α-gal epitopes have an appropriate conformation in order to bind anti-α-gal antibody. To assess this

PDFF, M2 and H9 cells were incubated in the presence of undiluted heat inactivated human serum, counter stained with an FITC conjugated anti-human immunoglobulin (Ig) antibody (1:200 - Southern Biotech) and binding assessed by flow cytometry (BD FACScan). Since human serum contains antibodies that may interact with antigens on the ES cell surface, but which are not α-gal, binding to wild type H9 cells was used as a baseline control. Additionally, to ensure that there was no non-specific binding of the secondary antibody, H9 cells were incubated in FITC-conjugated human Ig alone (Figure 3.2.15A).

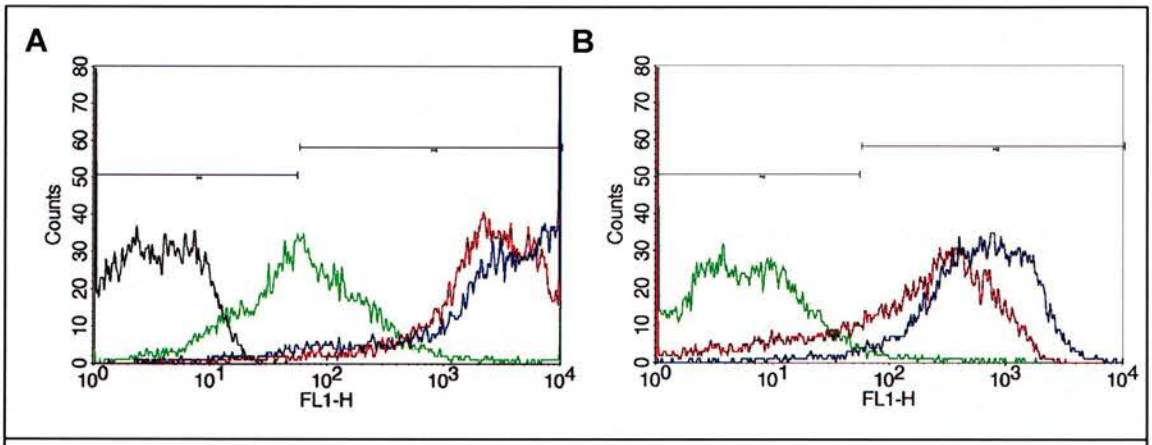


Figure 3.2.15: Transgenic human ES cells (M2-red) expressing the α-gal epitope are less efficient than endogenously expressing PDFF cells (blue) at binding human anti-α-gal antibodies. H9 (green), M2 and PDFF cells were incubated with undiluted heat inactivated (HIA) human serum and counter-stained with FITC conjugated anti-human immunoglobulin as a method of assessing appropriate conformation of the transgenic α-gal epitope. A) H9 cells incubated (green) and not incubated (black) with HIA serum, were used to determine the level of non-specific binding of the anti-Human Ig antibody, setting the baseline for B. B) Specific binding of human Ig to the α-gal epitope over and above the baseline.

Transgenic α-gal epitopes expressed on M2 cells were successfully able to bind natural anti-α-gal antibodies contained within human serum, although there was less

binding than was observed for PDFF cells, with 73% of cells binding compared to 95% (Mean fluorescence intensity 447.48 and 913.34 respectively: Figure 3.2.15B), suggesting that perhaps there was fewer epitopes on the M2 cells. As expected there was non-specific binding to wild type H9 cells, possibly due to the presence of anti-ES cell antibody contained within human serum, but most likely to be as a result of the sheer volume of protein contained within serum. When this non-specific binding was removed from the system, a substantial level of human Ig binding specifically to the α-gal epitope was observed. This suggested that M2 cells were likely to initiate a complement-mediated lysis response when in the presence of active human serum, however, binding of an antibody is only the first stage of complement mediated lysis, and the ability of complement to specifically lyse the M2 cells remained an essential part of this investigation.

3.3 Discussion

The use of TEG (trypsin/EGTA) as a method of disaggregation has been confirmed as an efficient and robust method for passaging undifferentiated human ES cells, facilitating monolayer growth while reducing spontaneous differentiation. Human ES cells passaged in this way continue to express the characteristic markers of human ES cells (SSEA-4, TRA-1-60 and TRA-1-81) and lack SSEA-1, characteristic of differentiating human ES cells. Although initial results appeared to indicate an increased incidence of chromosomal abnormalities, karyotypic analysis of a clonal cell line maintained with TEG dissociation for more than 60 passages, showed no evidence of genomic instability. Furthermore, the use of TEG has facilitated the use of techniques, such as flow cytometry, which require cells to be in single cell suspension. Since the group began work on optimising the culture conditions of human ES cells, the use of dispase (Gibco), a commercially available amino-endo peptidase produced by *Bacillus polymyxa*, to disaggregate human ES cells has been reported (Reubinoff, *et al.,* 2000). As with TEG the advantage of using dispase over collagenase IV and EDTA is that dispase yields a single cell suspension. Furthermore, dispase cleaves fibronectin, collagen IV, and to a lesser extent collagen I, and is therefore, less aggressive than both trypsin and collagenase IV in its disaggregation and does not disrupt the cell membrane, although, dispase is less specific than collagenase IV. It would be interesting in light of the results comparing TEG, EDTA and collagenase IV, to compare the dissociation of human ES cells using dispase or TEG and to compare their effects on plating efficiency and karyotypic stability.

3.3.1 The Use of H2-K^k as an Epitope for Complement-Mediated Lysis

The rational for using the murine MHC class I molecule, H2-K^k, as an epitope to initiate complement-mediated lysis stems from its successful application as a transgenic cell surface epitope on chicken blastodermal cells selected for by magnetic cell sorting (MACS) (Wei et al., 2001). However, to-date successful expression of this cell surface marker on cells of a human embryonic nature has not been reported. It is well known that MHC presentation is developmentally regulated (Warner and Gollnick, 1993), with low, lack or modified MHC expression being one of the suggested methods by which developing foetuses are protected from maternal immune responses (reviewed by Gaunt & Ramin, 2001). The low level of both MHC class I and β-2 Microglobulin (β-2M) expression on human ES cells reported by Drukker et al., (2002), was consistent with these views that the cells of the early embryo have some degree of immune privilege. It was hypothesised that since all MHC molecules require the association of β-2M to achieve stable transport to the cell surface, that one possible explanation for the lack of cell surface H2-K^k expression in transgenic H7 cells was insufficient β-2M to associate with both endogenous and transgenic MHC. However, over-expressing β-2M using a constitutive β-2M construct, Vcβ2 (a kind gift by Prof S Ono (University College, London) had no impact on the level of cell surface H2-K^k.

These data could be interpreted in a number of ways. Firstly, since there was no direct selection for the integration of Vcβ2, it was possible that the transfection was unsuccessful; selection of colonies was driven from the neomycin cassette contained in phTH2-KkpAPN. Secondly, that low levels of endogenous β-2M were not

responsible for the lack of cell surface expression of H2-K^k, but that there was a fundamental issue with translation of or transport of the H2-K^k protein, perhaps there is a genetic "safe guard" against presentation of MHC on embryonic cells. Thirdly, it was possible that the site of integration was silenced, although since the population was a pool this seemed unlikely.

As cell surface expression of the H2-K^k protein could not be achieved using a single transgene and could require co-transfection and induction to achieve expression, it was decided that this construct would be more complicated to use in a therapeutic setting, than α-gal.

3.3.2 The Use of Galα1-3Galβ1-4glcnac-R (α-Gal) as an Epitope for Complement-Mediated Lysis

By contrast to H2-K^k, engineering human ES cells to contain the α1,3galactosyltransferase (α-1,3Gal) gene was very successful. Cell surface expression of transgenic α-gal epitopes was found, in the best case, to be comparable with levels on endogenously expressing PDFF cells, as detected by binding of the α-gal specific lectin *BS-IB_4*. However, the level of α-gal expression was variable between clones, suggesting that the transgene was subject to positional effects. To some extent position effect variegation (PEV) was managed by maintaining the cells under constant G418 selection. When G418 selection was removed on the clonal cell lines, the number of cells expressing the α-gal epitope decreased. For the least variegated clone M2, the expression level fell from 95.40% as shown here to 82.60% (data not shown). However, for the F11 cell line, the drop in expression was more

dramatic, from 84.74% to 42.90%, as detected by flow cytometry. If selection had been relaxed permanently, there was the possibility that expression of the transgene would have been lost through silencing. A solution to this problem would be to target the α1,3Gal construct into the endogenous hTERT locus, so that α1,3Gal would collect the native hTERT promoter and consequently would be regulated appropriately avoiding silencing elements and heterochromatinisation. Alternatively, the construct could be targeted into a neutral genomic site, where expression patterns had been fully characterised. Such endogenous sites are being explored within the group by gene targeting (DiDomenico, et al., manuscript in preparation) and through the random integration and characterisation of an artificial locus (Thomson and Wojtacha, unpublished data). Alternatively, since the α1,3Gal construct is small, lentiviral transduction could be used to stably integrate DNA into the human ES cell genome, a technique that has been shown to be efficient and resistant to gene silencing (Martin et al., 2005; Gropp et al., 2003; Ma et al., 2003).

Despite the issue of variegation, preliminary functional data showed that cell surface α-gal epitopes on transgenic M2 cells were capable of binding natural anti-α-gal antibodies, to a similar level as the PDFF cells and it was therefore, anticipated that the epitopes would be able to initiate a complement mediated attack in line with that observed for endogenously expressing PDFF cells.

Importantly, from a therapeutic perspective, human ES cells which had integrated α1,3Gal behaved in the same way as their untransfected parental cells. Transgene integration did not affected their potentiality, with cell types representative of the 3

germ layers, endoderm, ectoderm and mesoderm, observed with both *in vitro* differentiation and teratoma formation *in vivo* when cells were injected intramuscularly into SCID mice. Furthermore, the M2 cell line maintained a stable 46 XX karyotype after over 60 passages.

3.4 Conclusion

The results presented in this chapter, show that the use of trypsin/EGTA (TEG) as a method of disaggregating human ES cells for routine culture has allowed the generation of a more homogeneous population of human ES cells, with little, if any differentiation. That ES cells cultured in this way, dissociate into a single cell suspension, aiding optimisation of electroporation techniques, resulting in efficient introduction of transgenes with greater success than reported by Zwaka and Thomson, (2003).

For reasons that are not yet clear, transgenic expression of H2-K^k was not achieved, despite successful integration of the transgene. However, human ES cells were engineered to express high levels of α-gal epitopes through the random integration of a hTERT/α1,3Gal transgene. Expression of α-gal epitopes as a consequence, was similar to that observed on endogenously expressing cells (PDFF) and preliminary data suggested that the expressed epitopes were able to bind natural anti-α-gal antibodies found in human serum. It was therefore, hypothesised that the α-gal epitope would successfully initiate complement-mediated lysis when cells were exposed to active human serum.

CHAPTER 4 OPTIMISING COMPLEMENT-MEDIATED LYSIS FOR HUMAN ES CELLS.

4.1 Introduction

Complement is a component of the innate immune system, involved in self-non-self recognition. Activation of complement via the classical pathway (see section 1.7.1.2) is dependent upon binding of antibody to antigen, resulting in the exposure of complement binding sites in the Fc portion of the antibody. The ovine foetal fibroblast cell line, from the Polled Dorest breed (PDFF), like many other cells, endogenously expresses the α1,3galactosyltransferase (α1,3Gal) gene, and hence expresses the α-gal epitope on the surface of their cells (Figure 3.2.12). Through evolutionary time humans have acquired a fixed mutation in the α1,3Gal gene resulting in loss of gene function, as previously discussed, and have developed natural anti-α-gal antibodies as a result of the continued presence of the gut microflora, which express α-gal (Galili *et al.,* 1988b). Therefore, human serum not only contains all of the components of the complement cascade required to cause cell lysis, it also contains a high titre of natural anti-α-gal antibody, up to 1% of total circulating immunoglobulin (Ig) (Galili *et al.,* 1987b), to trigger it. Together, these qualities provided an opportunity to establish optimal working conditions for complement-mediated lysis experiments, independent of the effects of protein expression levels and variegation from transgenic cell lines.

4.1.1 Chapter Aims

1. To become familiar with established protocols for the detection of cytolytic activity using the Calcein-release technique.

2. To optimise the Calcein-release assay to provide a sensitive and reproducible method for the detection of cytolytic activity.

3. To determine the sensitivity of human ES cells to complement-mediated lysis.

4.2 Results

4.2.1 Using Established Methods for the Detection and Quantification of Complement-Mediated Cell Lysis Utilizing the α-gal Epitope and Endogenous Antibodies as a Trigger.

PDFFs were loaded with 2μM Calcein.AM in accordance with the methods of Spiller (2000) for an hour at 37°C prior to lysis. Lysis was performed with 250μl of 50% human or foetal bovine sera diluted in gelatin veronal buffer plus magnesium and calcium (GVB^{2+}), for 1 hour at 37°C, to determine the specificity and sensitivity of the reported conditions for the Calcein-release assay. Initially, results appeared encouraging (Figure 4.2.1), significant Calcein-release was only observed in the presence of active human serum, suggesting that although the level of specific lysis (Calcein-release from active serum minus that from heat inactivated serum) was lower than expected (between 40-50%), the α-gal epitope was capable of eliciting a complement mediated response triggered via natural anti-α-gal antibodies contained within human serum.

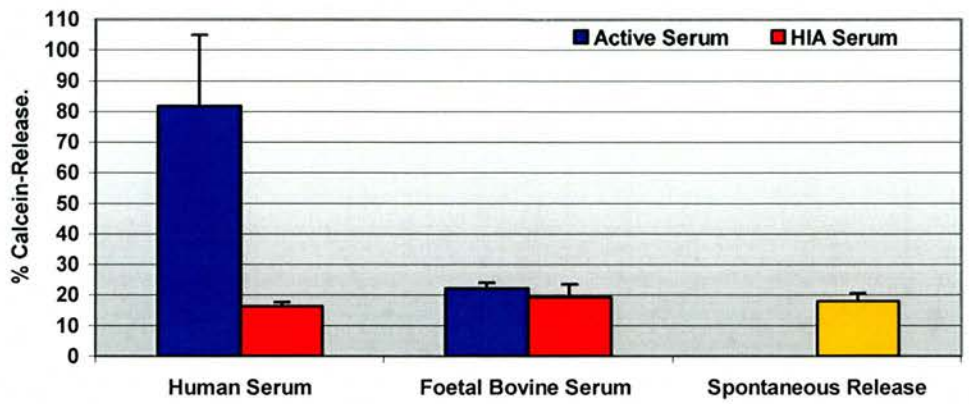


Figure 4.2.1: Preliminary data suggested that serum lysis of PDFF cells appeared to be specific to active (blue) human serum. Incubation with active foetal bovine serum and heat inactivated (HIA) sera (red) resulted in the same level of Calcein-release as seen spontaneously (yellow). Data represents the mean of 6 replicates and error bars represent the standard deviation of the individual observations.

However, when these conditions were tried on both wild type (H9) and transgenic (M2) human ES cells, high levels of cell death were observed in the heat inactivated serum control as well as in the H9 cells, which did not present a lysis epitope, suggesting that the conditions of this assay were not optimal for human ES cells (Figure 4.2.2).

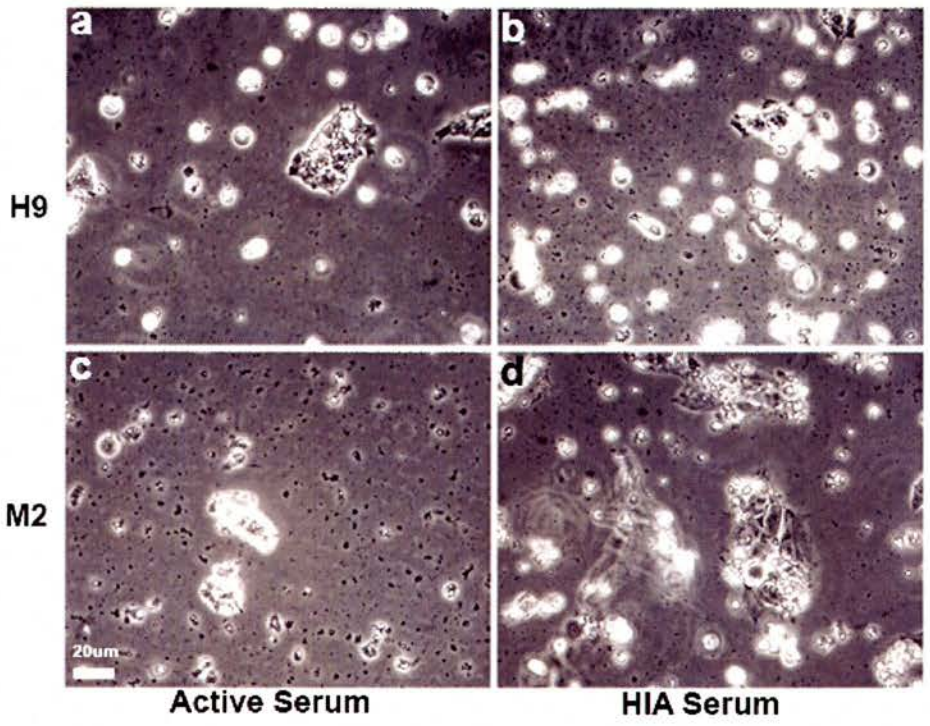


Figure 4.2.2: Human ES cells are non-specifically killed using established conditions. Serum lysis experiments using reported protocols (Spiller, 2000) on H9 (a-b) and M2 (c-d) human ES cells revealed a high level of non-specific cell death in the presence of both active (a&c) and heat inactivated (b&d) human serum. Cells were suspensded at $1x10^6$ in 250µl of human serum diluted to 50% in GVB^{2+} buffer, and incubated at 37°C for 1 hour, before being re-plated in standard tissue culture conditons. Cell Survival was assessed 24 hours later based on plating density. All images were view under x40 objective, scale bar represents 20um.

4.2.2 Different Assay Buffers have a Dramatic Effect on the Level of Spontaneously Released Calcein from Cells in the Absence of Complement.

To investigate the possibility that a component of the assay was responsible for the high level of non-specific cell death, each element was individually assessed. Human ES cells are cultured under strict conditions, requiring very specific medium, in comparison to that of fibroblastic cell types, and are highly sensitive to changes in

those conditions. Therefore, the assay buffers were the obvious components of the complement-mediated lysis assay to be addressed.

During Calcein.AM loading the cells were incubated in culture medium. However, during lysis, the suggested serum dilution buffer was veronal buffered saline (VBS). The necessary component of this buffer is calcium, since initiation of the classical complement pathway requires the presence of calcium (Kosasi, *et al.,* 1989). However, although VBS is a saline, the salt concentration was such that the buffer was hypotonic, which consequently induced an osmotic pressure gradient across the cells leading to cell swelling and eventual cell death. The addition of gelatin to VBS (GVB^{2+}) was recommended for use with cell types that were highly sensitive to lysis for maintenance of osmolarity (Spiller, B. Pers. Com.). Despite the use of GVB^{2+} the level of non-specific cell death remained high when human ES cells were exposed to diluted serum (Figure 4.2.2). To test the hypothesis that the buffer was responsible for high background cell death, human ES cells were loaded with Calcein.AM as previously described, and then incubated in neat GVB^{2+} buffer, PBS plus magnesium and calcium, or complete culture medium for an hour at 37°C, before recording the level of spontaneous Calcein-release in the supernatant. Interestingly, culture in isotonic PBS (plus Mg^{2+}/Ca^{2+}) resulted in elevated levels of spontaneous Calcein-release (53%) compared to just 27% when cells were incubated in GVB^{2+}, confirming that the presence of gelatin reduces the susceptibility of cells to lysis in a hypotonic environment. As expected, however, the incubation of cells in culture medium reduced the level of spontaneous release to just 15%, suggesting that the cells were less stressed (Figure 4.2.3).

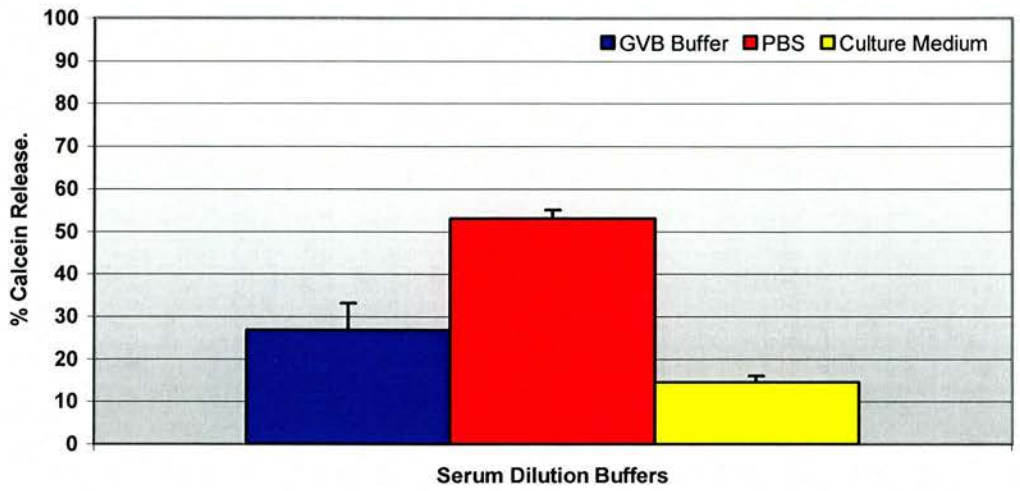


Figure 4.2.3: Serum dilution buffer contributed significantly to the observed spontaneous Calcein-release. Background Calcein-release resulting from incubation with the suggested serum dilution buffer, GVB^{2+} buffer (blue), was 27%. When cells were incubated with PBS (red) was this background increased, as expected, to 53%. The use of culture medium (yellow) reduced the background to 15%, without increasing the level of auto-fluorescence. Data represent the mean of triplicate results and error bars represent the standard deviation of the individual observations.

While incubation in GVB^{2+} alone produced considerably higher levels of spontaneous release than incubation in culture medium, these levels were not as high as anticipated, and were in fact only 7% higher than the accepted level for spontaneous Calcein-release, reported in the literature (Phelps et al, 2003). However, it was clear that incubation in GVB^{2+} was sufficient to compromise the ability of H9 cells to re-plate (Figure 4.2.4), helping to explain the high levels of non-specific lysis observed (Figure 4.2.2).

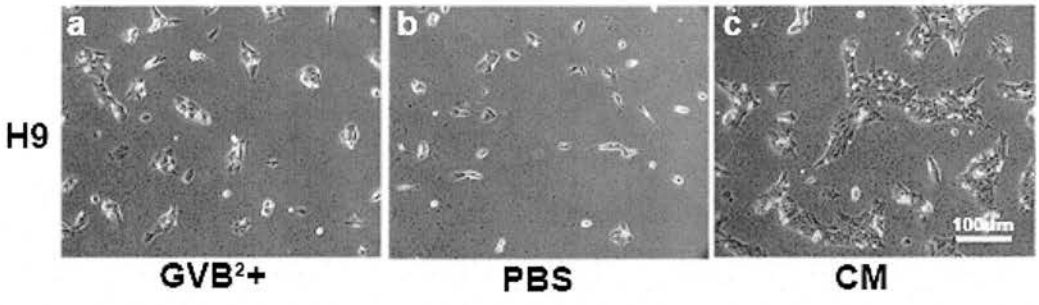


Figure 4.2.4: Cell survival following incubation with culture medium was significantly better then incubation with GVB^{2+} or PBS plus. 10^5 H9 cells were incubated with GVB^{2+} (a), PBS (b) or conditioned medium (c) for an hour before being re-plated. Cells were allowed to attach and were assessed for cell survival based on plating density. Images a-c are representative examples of cell density 24 hours later.

Complement is a series of proteins with short half-lives, which are rapidly degraded as a mechanism of self-regulation (Liszewski *et al.,* 1996), so it was essential to determine that initiation of a complement cascade could and would still occur if the serum was diluted in culture medium and not GVB^{2+}. PDFF, M2 and H9 cells were loaded with Calcein.AM (2µM for 1 hrs at 37°C) and incubated in 250µl of active or heat inactivated human serum, diluted to 50% in the appropriate culture medium using a modified version of the published method (Spiller, 2000). A culture medium alone control was included to determine the level of spontaneous Calcein-release. Cells were incubated in suspension at 37°C in a humidified incubator plus 5% CO_2, for an hour.

The dilution of serum in culture medium instead of GVB^{2+} had no impact on the initiation of a classical complement attack (Figure 4.2.5). 40% of the PDFF cells died, specifically as a result of the active serum, as previously observed (Figure

4.2.1), although, the level of non-specific lysis increased for the PDFFs from 21% to 37%. Furthermore, levels of spontaneous release were in excess of the accepted 20% background. Nevertheless, a substantial increase in the level of cell survival was observed for cells expected to survive complement-mediated cell lysis (H9) in both active and heat-inactivated sera to approximately 70%. Unfortunately, the same level of cell survival was also observed in the transgenic M2 cell line (Figure 4.2.5), suggesting that they were not susceptible to complement-mediated cell lysis.

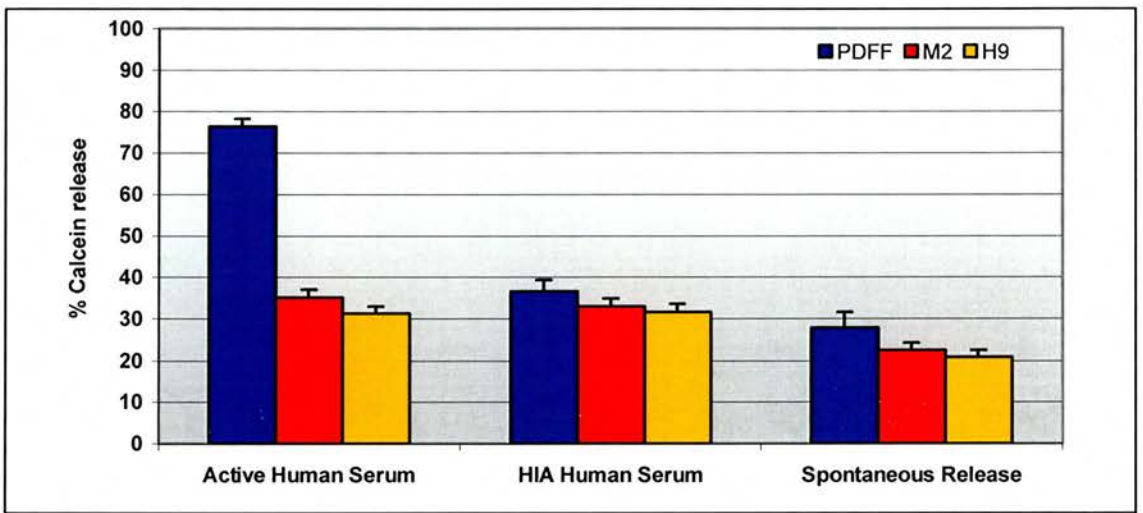


Figure 4.2.5: Using culture media, as serum dilution buffers, had no impact on complement initiation. Specific PDFF lysis (blue) was observed at a level of 40% the same as when GVB^{2+} was used. Furthermore, there was no specific lysis observed in the H9s (yellow) as expected, however, neither was there any lysis observed for the transgenic M2 cell line (red). Bars represent the mean of triplicate samples and the error bars represent the standard deviation of the individual observations.

Confirmation of these results was, however, difficult to achieve due to significant inter-experimental variation (Figure 4.2.6). From at least four independent assay runs, the average level of specific PDFF lysis ranged from 7% to 40%, and

interestingly the range of specific M2 lysis was similar, from 0% to 33%, although unlike the PDFFs, specific M2 lysis was only observed on one occasion, giving a value of 33%. The level of specific H9 lysis, although variable was within a much smaller and more acceptable range, from –2 to 4% (Figure 4.2.6).

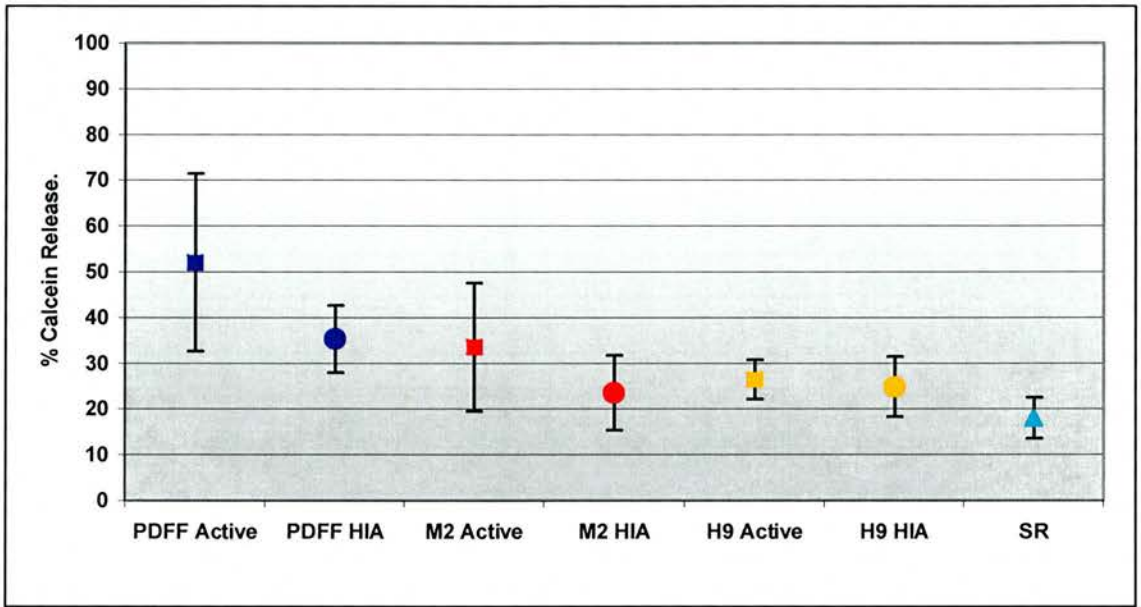


Figure 4.2.6: Inter-experimental variation was very high. The reported conditions for the detection of complement-mediated lysis through Calcein-release (Spiller, 2000) were highly variable between runs. Cells were loaded with 2μM Calcein.AM for an hour at 37°C before incubation with active or heat inactivated (HIA) serum or culture medium (SR). The value for SR reflects the mean of all cell types. All other values are the mean percentage Calcein-release from triplicate experiments and error bars represent the standard deviation of the individual observations.

The high inter-experimental variation, but absence of even low levels of reproducible M2 lysis led to the formation of two working hypotheses, either a) the experimental conditions for the use of the Calcein-release assay were still not optimal or b) that because of their embryonic origin, human ES cells expressed high levels of

complement regulatory proteins and were hence resistant to complement-mediated attack.

4.2.3 Human ES cells Express High Levels of Complement Regulatory Proteins CD46, CD55 and CD59.

The initial lack of specific M2 lysis, using the published protocol, suggested that human ES cells could be resistant to complement-mediated lysis. It was hypothesized that this may be due to their embryonic nature, and possible over-expression of complement-regulatory proteins (Simpson et al., 1993). Therefore, the expression of CD46 (Membrane Co-factor protein MCP), CD55 (Decay accelerating factor DAF) and CD59 (Membrane inhibitor of reactive lysis MIRL), the three major membrane bound complement regulatory proteins, were assessed by flow cytometry. Expression of complement inhibitors on human ES cells was compared to known expression levels on cell types sensitive to complement-mediated lysis, human multipotential erythroid progenitor (K562) cells and human promonocytic (U937) cells (Jurianz *et al.,* 2001; von Zons *et al.,* 1997; Van den Berg *et al.,* 1994) and a second cell type of embryonic origin, human embryonic kidney, HEK 293, cells.

Pre-conjugated antibodies (Table 4.2.1) were used to stain K562, U937, HEK 293, H1, H7 and H9 cells (10^6), in staining buffer (PBS with 2% FBS and 2mM EDTA), on ice for 30 minutes in the dark. Flow cytometry was performed using a FACScan (Becton Dickenson), and CellQuest Pro analysis software. Analysis of variance (ANOVA) was performed (Springbett, A., Roslin Institute, Edinburgh) to determine the significance of shifts in the mean fluorescence intensity between cell types.

Antibody Name	Fluorochrome	Dilution	Suppliers
CD46 (MCP)	FITC	0.1mg/ml diluted 1:10	Serotech
CD55 (DAF)	PE	0.2mg/ml diluted 1:10	BD Bioscience
CD59 (MIRL)	FITC	0.5mg/ml diluted 1:10	BD Bioscience

Table 4.2.1: Complement regulatory antibodies, fluorochrome, dilution factors and suppliers for the antibodies used for flow cytometry.

Human ES cells expressed statistically higher ($P<0.01$) levels of CD46 than K562 (Figure 4.2.7a), yet statistically lower levels than U937, with the exception of H1 cells, which express more CD46 than all of the other cell lines (Figure 4.2.7b). Expression of CD46 on the HEK 293 cells followed that of human ES cell line H9 (Figure 4.2.7a). CD55 expression on the other hand, was significantly elevated on all human ES cells and HEK 293 cells ($p<0.01$) compared to levels expressed by both K562 and U937 (Figure 4.2.7c). CD55 expression was expressed on H1 and H7 cells in similar amounts however, H9 cells (the parental cell line to M2 cells) expressed significantly ($P<0.01$) more CD55 than both H1 and H7 (Figure 4.2.7d).

It is reported in the literature, that K562 cells have high levels of complement inhibitory protein CD59 (Jurianz *et al.,* 2001), which was confirmed here (Figure 4.2.7e). CD59 expression on HEK 293 and human ES cells was statistically lower than on K562 cells, but its expression was statistically higher than on U937 cells. All three human ES cells lines expressed CD59 in equal amounts (Figure 4.2.7f).

Whilst human ES cells express significantly higher levels of complement regulatory protein CD55 and CD59 statistically, the biological significance of these elevated

levels remained unclear. It was decided that in order to answer this question, it was first necessary to determine the optimal working conditions for both *in vitro* complement-mediated lysis and the Calcein-release detection-assay.

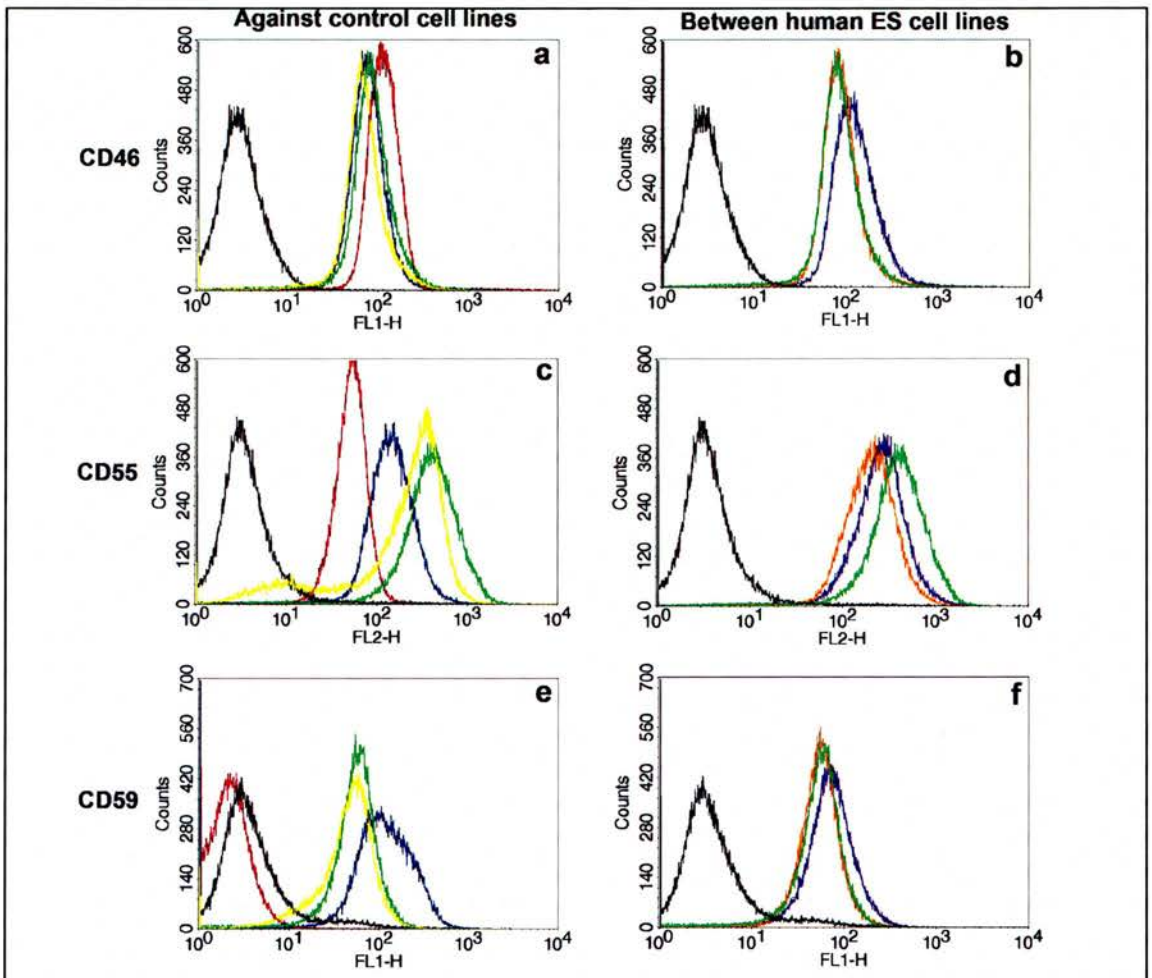


Figure 4.2.7: Expression levels of complement regulatory proteins, CD46 (A&B), CD55 (C&D) and CD59 (E&F) by flow cytometry. A, C and E compare expression levels of K562 (blue), U937 (red) HEK 293 (yellow) and H9 (green) cells. B, D, and F compare expression levels between different human ES cell lines H1 (purple), H7 (orange) and H9 (green). In all cases the black line represent the fluorescence from an isotype control.

4.2.4 Assessing the Kinetics of Calcein.AM Loading

The Calcein-release assay has been reported by many (Iwanowicz *et al.,* 2004; Phelps *et al.,* 2003; Neri *et al.,* 2001; Oral *et al.,* 1998; Braut-Boucher *et al.,* 1995; Lichtenfels *et al.,* 1994) to be a reliable and reproducible alternative to the ^{51}Cr-release assay. It was therefore, decided to optimise the Calcein-release assay, specifically for assessment of complement-mediated lysis of human ES cells.

Initially, Calcein concentration and loading time were determined for PDFF's. Serial dilutions of Calcein.AM were made in culture medium covering a range of concentrations reported in the literature, 0, 2, 5, and 10μM (Iwanowicz *et al.,* 2004) and in further excess, 20, 40 and 80μM. PDFF's ($1x10^5$) were resuspended in 50μl of diluted Calcein.AM and incubated for 1, 2 and 4 hours at 37°C in a humidified incubator plus 5% CO_2, in an attempt to find a point of cell saturation. Following Calcein loading, the cells were then assessed for the level of spontaneous release (SR) through a further 1-hour incubation with 100μl culture medium, or for the level of total release (TR) through incubation with the same volume of 0.1% triton-X 100 (Figure 4.2.8) using the protocol described by Iwanowicz *et al.,* (2004).

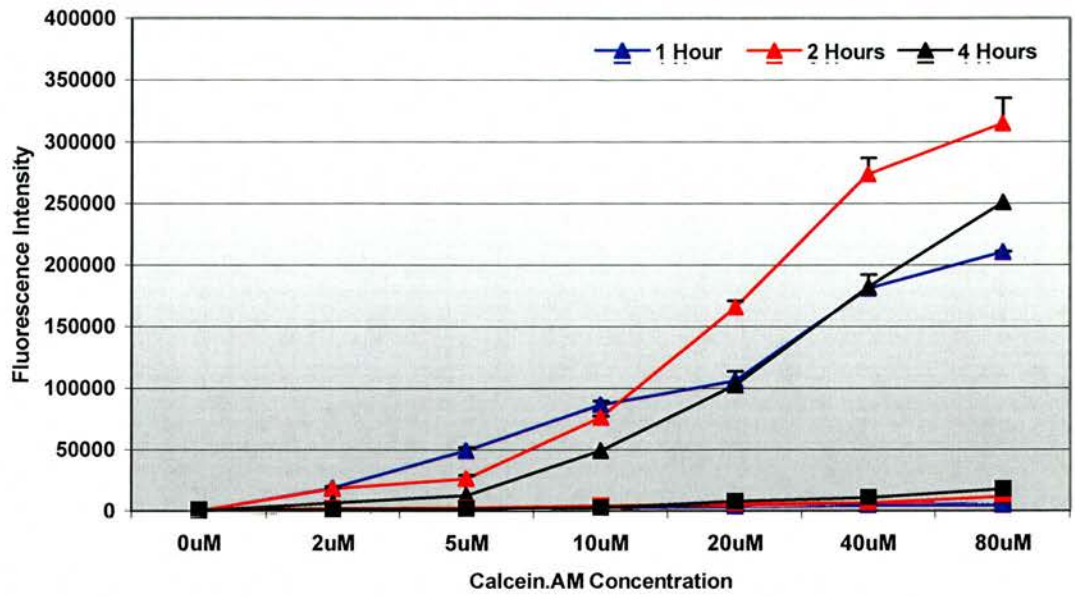


Figure 4.2.8: Calcein.AM concentrations required for labelling $1x10^5$ PDFF cells in suspension far exceed those reported in the literature for other cell types. Using established protocols (2μM) there was little difference between the level of spontaneous release (■) and total release (▲). However, at 10μM and above there is clear separation. Incubation for an hour (blue) seems efficient at the lower concentrations but above 10μM 2 hours (red) gives a more significant separation. 4 hours (black) in the presence of Calcein.AM appears to reduce the level of total release, and increase slightly the level of spontaneous release, possibly due to a toxic effect. The data represent the mean of 3 independent replications; error bars show the standard error about the mean.

It was clear from these data that loading PDFF's with 2μM Calcein.AM was insufficient to reliably differentiate between spontaneous (■) and total (▲) release (Figure 4.2.8). Furthermore, since the cells had not reached saturation at 80μM, a 40-fold increase in Calcein.AM, it was evident that the use of 2μM was resulting in the majority of cells containing no label, and thus reducing the assays sensitivity as small errors in cell counting could have accounted for the observed variation in

fluorescence intensity readings, and consequently percentage Calcein-release between assay runs.

Interestingly, when cells are loaded with low concentrations of Calcein.AM, up to 10μM, a 1-hour incubation appeared to provide maximal Calcein.AM uptake, giving the highest values of total release. However, for concentrations of 20μM and above, a 2-hour incubation provided significantly higher levels of Calcein.AM loading. Furthermore, the level of spontaneous release (SR) following 1- and 2-hour loading was comparable, whereas SR was slightly elevated following 4 hours of loading. Together with the reduced Calcein.AM uptake following a 4-hour incubation these data suggested that prolonged incubation in Calcein.AM was detrimental to cell survival. It was therefore decided, that a loading time of 2 hours be used to optimise the concentration of Calcein.AM required to achieve saturation for different cell types, without causing increased levels of background through non-specific cell death.

4.2.5 Cell Types Vary in their Ability to Take-Up Calcein.AM and in their Capacity to Retain the Fluorescent form of Calcein.

To determine if Calcein.AM loading and retention varied for different cell types, PDFF, LLC-PK1 (porcine kidney epithelial cells) and wild type H9 ES cells were incubated with serial dilutions of Calcein.AM, extending to 160μM, for 2 hours at 37°C in a humidified incubator plus 5% CO_2,

PDFF cells were able to take up and retain the Calcein.AM label more efficiently than either human ES cells or LLC-PK1 cells (Figure 4.2.9). At concentrations of 10μM there was already a clear difference between the level of spontaneous and total release. Likewise for human ES cells, uptake of Calcein.AM was clearly detectable at concentrations as low as 10μM, however, the level of spontaneous release was significantly higher then in PDFF's reducing assay sensitivity. Therefore, in order to achieve maximal separation of spontaneous and total released Calcein from human ES cells it was decided that a concentration of 40-80μM Calcein.AM was necessary (Figure 4.2.9).

Interestingly, LLC-PK1 cells were more resistant to Calcein labelling than both PDFF and human ES cells. Calcein uptake remained low until the Calcein.AM concentration had exceeded 40μM. However, LLC-PK1 cells were better at retaining the Calcein label than human ES cells, but not as efficient as PDFF cells (Figure 4.2.9).

A Calcein.AM concentration of 160μM had a negative impact on the level of total release in both PDFF and human ES cells, and on the level of spontaneous release in human ES cells, suggesting that at high concentrations Calcein.AM was toxic to these cell types, preventing retention of the label during the labelling process and hence reducing the level of Calcein released, even at the spontaneous level (Figure 4.2.9). LLC-PK1 cells however, were not adversely affected by the high Calcein.AM concentration, suggesting that these are potentially more resilient cells.

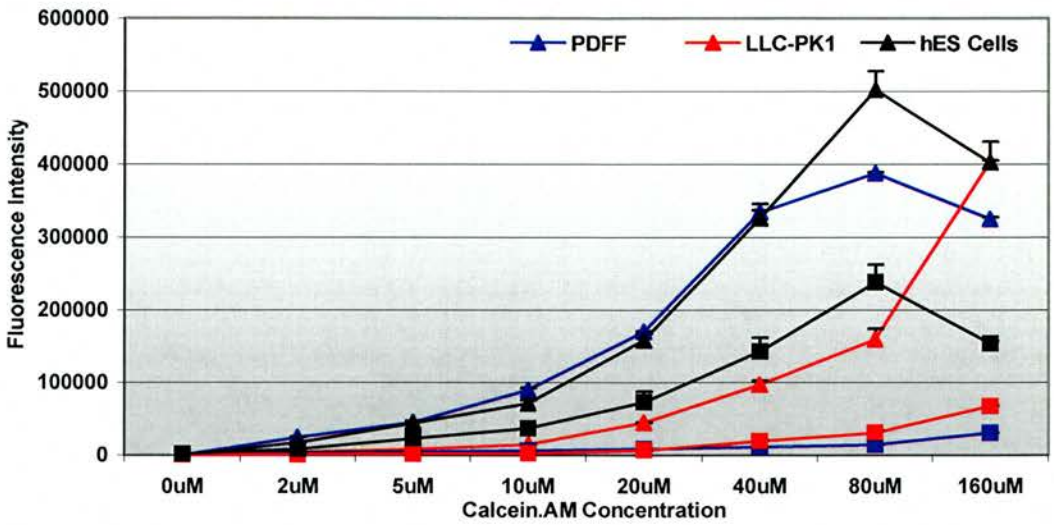


Figure 4.2.9: Cell types varying in their ability to take up Calcein.AM and retain Calcein. PDFF cells (blue) were the most receptive to Calcein.AM labelling with an obvious difference between spontaneous (SR ■) and total release (TR ▲) being observed at concentrations as low as 10μM. Human ES cells (black) were as receptive as PDFF (blue) to Calcein.AM loading, at concentrations of 10μM and above, however, they were the least able to retain the label, with high levels of spontaneous release also observed at 10μM. LLC-PK1 cells (red) were the least receptive to labelling with Calcein.AM with no distinguishable uptake observed until concentrations exceed 40μM. Data represents the mean of duplicate experiments; error bars represent the standard error about that mean.

PDFF cells were found to mimic more closely the human ES cells in their receptiveness to Calcein loading, compared with the LLC-PK1 cells, and so PDFF cells were used as the positive control cell line in all remaining experiments. The reduced ability of human ES cells to retain the Calcein label resulted in the use of a higher Calcein concentration (40μM) for both human ES and PDFF cell labelling (Figure 4.2.9).

4.2.6 Increasing the Calcein.AM Concentration Significantly Improved the Reproducibility of the Calcein-Release Assay.

To determine whether insufficient Calcein loading had played a significant part in the low sensitivity of the Calcein-release assay, PDFF cells were loaded with Calcein.AM (10^5 cells in 50µl) diluted to 2µM and 40µM in culture medium (2hrs at 37°C), before being incubated with either active or heat inactivated (HIA) serum, diluted to 50% with culture medium, culture medium alone (SR) or 0.1% Triton-X 100 (TR) and the Calcein-release recorded using the method described by Iwanowicz *et al.,* (2004).

By increasing the concentration of Calcein.AM used to label PDFF cells to 40µM, the level of specific lysis increased 43% over that for the 2µM concentration, from 24% to 67% specific lysis (Figure 4.2.10). Furthermore, using the method reported by Iwanowicz *et al.,* (2004), the level of spontaneous release and background lysis was also reduced, to just over 10% (Figure 4.2.10) for 2µM Calcein.AM, compared with excess of 20% observed using the method of Spiller (2000) (Figure 4.2.5) and concurrent reduction in the level of intra-experimental variation (Figure 4.2.11).

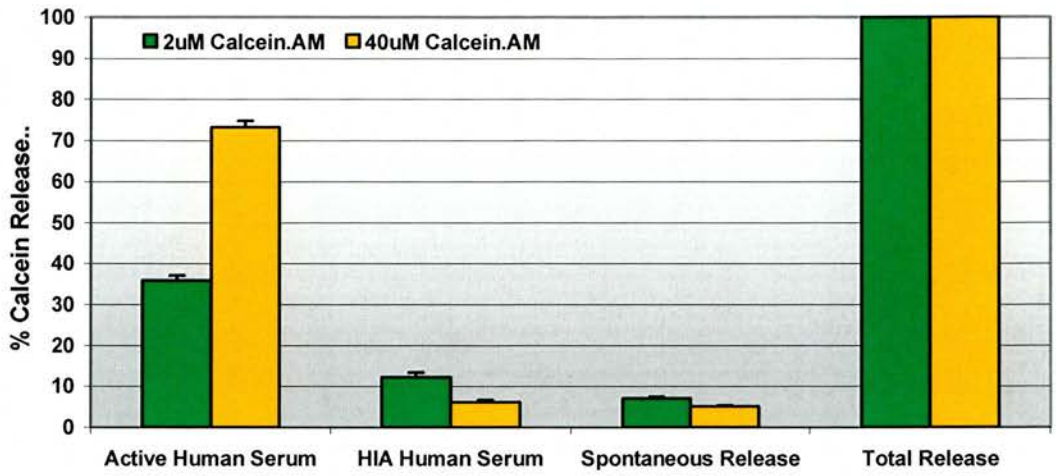


Figure 4.2.10: Increased Calcein concentration significantly improves the ability to distinguish between specific and background lysis. The method reported by Iwanowicz *et al.*, (2004), also reduced the level of background release for both 2µM (green) and 40µM (yellow) to less than 20%, and reduced intra-experimantal variation compared with the method of Spiller (2000). Data is representative of three independent replicats; error bars represent the standard deviation of the individual observations.

The methods of Iwanowicz *et al.* (2004) and Spiller (2000) only differed subtly but those differences had a significant impact on the reproducibility of the Calcein-release assay, particularly as the technique was being performed inside a laminar flow-hood. Instead of using 250µl volumes of serum, medium or detergent, as described by Spiller, Iwanowicz *et al.*, reported the use of 100µl volumes using 10^5 cells, instead of 10^6 (Iwanowicz *et al.*, 2004; Spiller 2000). However, of greater significance was that while Spiller suggested the collection of all 250µl of supernatant, to record the level Calcein released, Iwanowicz *et al.*, reported the collection of a 50µl sample, which was then mixed with 2x Triton-X 100 and recorded (Iwanowicz *et al.*, 2004; Spiller, 2000). By only removing a sample of the supernatant, after the surviving cells and cellular debris had been pelleted, there was

a significantly reduced risk of contaminating the measurement of fluorescence in the supernatant by bright Calcein fluorescence from any surviving cells. The risk of this contamination with the method reported by Spiller (2000) was high, and would have accounted for the high levels of intra-experimental variation observed (Iwanowicz *et al.,* 2004; Spiller, 2000). This hypothesis was confirmed when both experimental designs were used in parallel. However, despite the reduced intra-experimental variation, by far the most significant improvement in reliability was as a result of increasing the concentration of Calcein.AM used to load PDFF cells. Loading of PDFF cells with 40μM Calcein.AM considerably reduced the inter-experimental variation, providing a clear distinction between specific and background lysis (Figure 4.2.11) improving the assays sensitivity and importantly reproducibility in independent experiments.

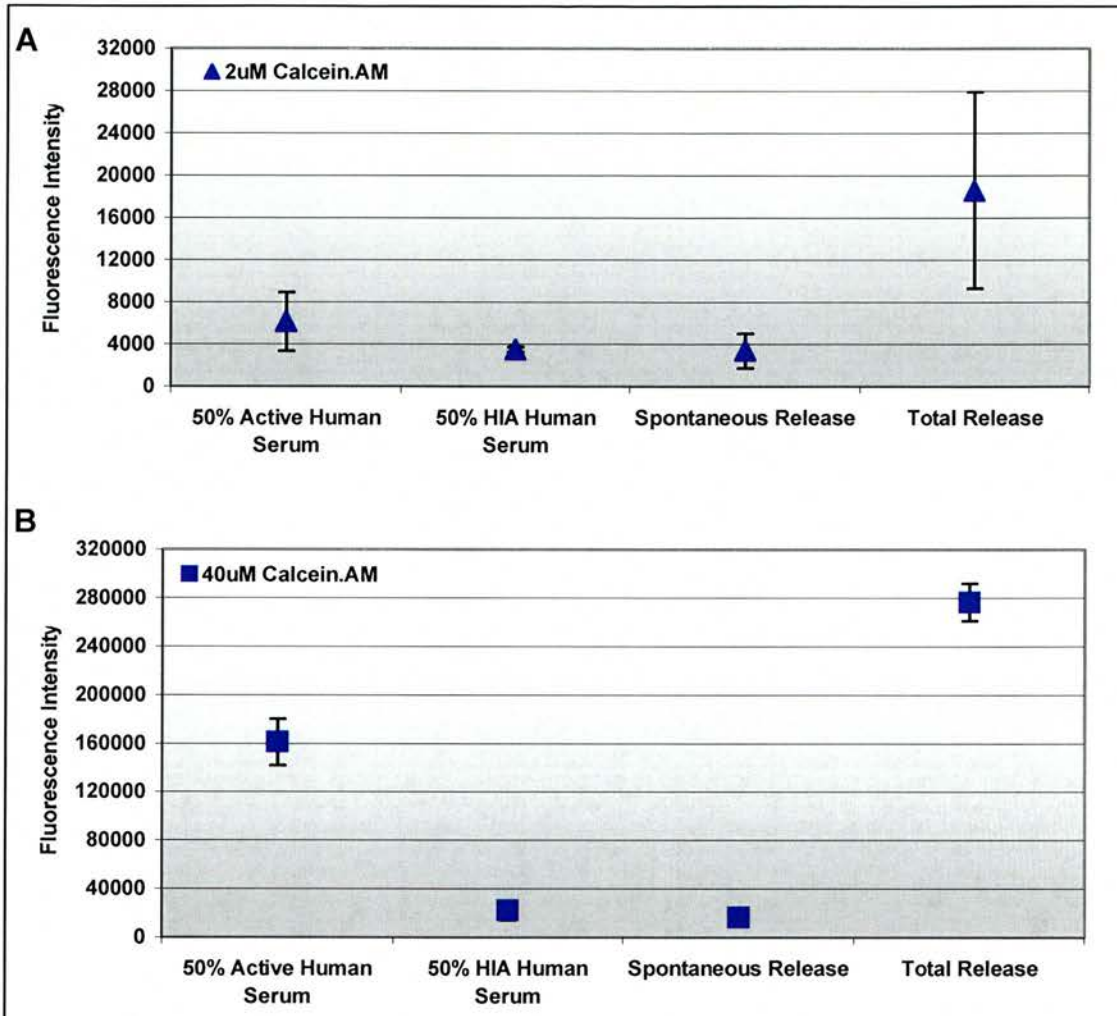


Figure 4.2.11: Inter-experimental variation is significantly reduced with increased Calcein.AM loading. PDFF cells were loaded with Calcein.AM at A] 2μM and B] 40μM and then cultured appropriately. There was a significant reduction in the range of values for any one of the treatments when 40μM were used compared to 2μM. Furthermore, there was increased sensitivity between the level of Calcein released as a result of active and heat inactivated (HIA). Data shows the variation across three independent replicates; error bars represent the standard deviation of the individual observations.

However, while the reproducibility of the assay had significantly improved, there was still a degree of variation that remained constant despite the increased

Calcein.AM concentration. This variation was seen repeatedly following incubation with active serum and may simply be the result of variability in the level of active complement in the different serum aliquots since all other treatments are independent of active lysis.

Of greater concern was the relatively low level of specific PDFF cell lysis. Based on evidence in the literature for xenograft rejection, as a result of the presence of the α-gal epitope (Unfer *et al.,* 2003) the level of lysis of PDFF cells with human serum remained noticeably lower than anticipated at best producing 60% cell lysis. For this technique to be useful for removal of contaminating humans ES cells, it was necessary to improve the efficiency of cell lysis.

Reports in the literature have suggested that the use of frozen or lyophilised serum reduces cytolytic activity to approximately 60%, while fresh serum gave lysis in excess of 90% (Phelps *et al.,* 2003), suggesting that the current assay conditions could be further refined by collecting fresh serum as a source of complement and titrating the serum concentration to obtain optimal cytolytic activity.

4.2.7 Serum Concentration, Source and Storage have a Significant Influence on its Complement Cytolytic Activity.

Previously the source of serum had been a pooled sample of blood group-A donors, purchased from Harlan Sera Labs (Loughbourgh, UK), which arrived frozen at -20°C and was then subsequently dispensed into small aliquots and frozen at -80°C until required. Serum from this source will be referred to as commercial human serum.

As a comparison, fresh serum collected from a pool of blood group-A donors (Roslin Institute, Edinburgh), was either used directly as fresh serum, or frozen immediately in small aliquots at -80°C for future use. Serum from this source will be referred to as either fresh or frozen human serum respectively.

PDFF cells (10^5) were loaded with 40μM Calcein.AM for 2 hours at 37°C. They were then incubated with 100μl of undiluted active or heat inactivated (HIA) fresh, frozen or commercial human serum. As controls, cells were incubated with culture medium or 0.1% triton-X 100 alone to assess the level of spontaneous (SR) and total release (TR) respectively.

Incubation with both fresh and freshly frozen human serum significantly increased the level of Calcein-release, compared with commercial serum, by over 30% (Figure 4.2.12), while the level of Calcein-release from heat inactivated (HIA) serum remained constant between all the sera. Interestingly, when commercial serum was stored as instructed, at -20°C, the cytolytic activity was even further reduced after just 2 months (Figure 4.2.13), confirming the instability of the complement proteins.

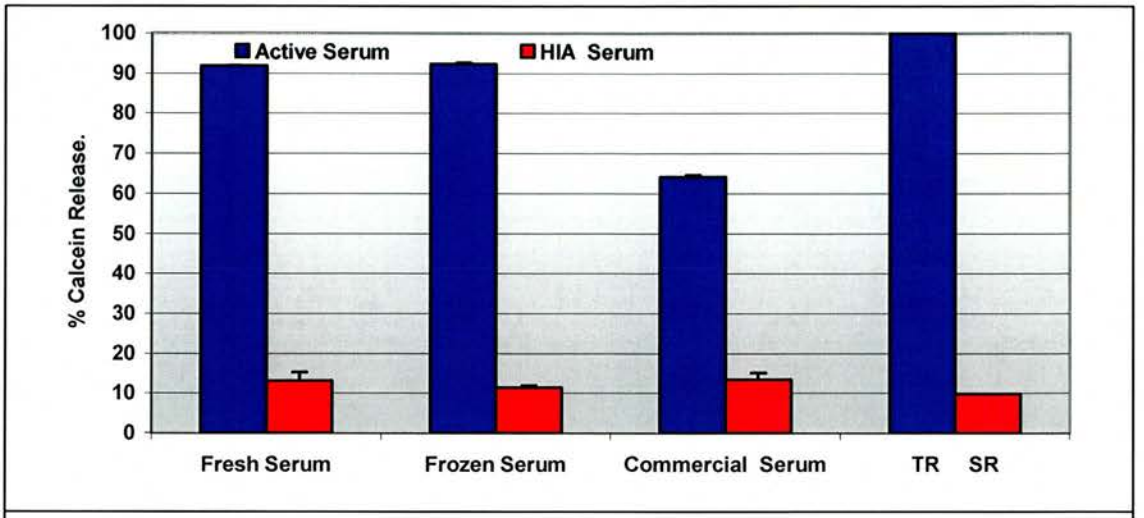


Figure 4.2.12: Serum frozen directly at -80°C has the same cytolytic activity as freshly acquired serum, which is some 30% more active than commercial serum. Active (blue) fresh and frozen serum caused over 80% specific Calcein-release, compared with 50% from active commercial serum. Heat inactivation (red) of all serum produced levels of Calcein-release comparable with those observed for spontaneous release (SR). Data shows the mean of two independent replicates; error bars represent the standard deviation of the individual observations.

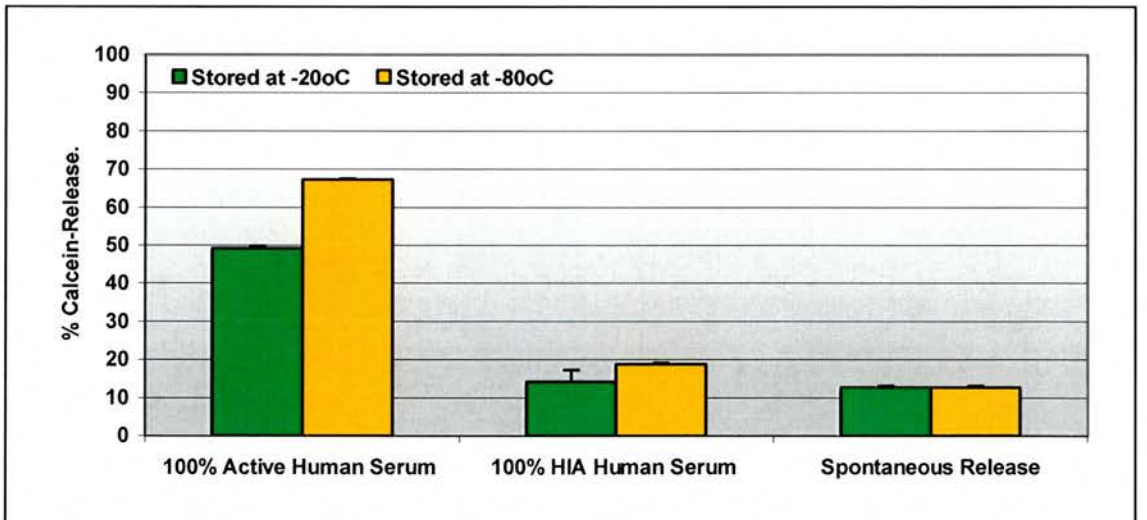


Figure 4.2.13: The cytolytic activity of commercial serum is further reduced following prolonged storage -20°C. PDFF cells loaded with 40μM Calcein were lysed with the same batch of commercial serum, which had been stored at -20°C (green) or -80°C (yellow) for a period of 2-months and clearly shows a degradation of the cytolytic active. Error bars represent standard error of the mean of 6 replicates.

In previous experiments, the assumption was that high concentrations of serum would be deleterious to the cells in a non-specific manner, and therefore, the serum was diluted to 50% in culture medium. To test this assumption, PDFF cells (10^5) were loaded with Calcein.AM as previously described and then incubated with fresh human serum diluted to 2, 5, 10, 20 and 50% in culture medium or incubated in neat serum (100%), both active and heat inactivated, for an hour at 37°C. Included in the experiment were culture media alone and 0.1% triton-X 100 controls to assess the level of SR and TR respectively.

Surprisingly, when cells were incubated in serum at a concentration less than 50%, there was no significant difference between the levels of Calcein-released due to active or heat inactivated serum, which were not significantly different to the level of Calcein released spontaneously (Figure 4.2.14A). At 50% serum, there was an average of 40% specific PDFF lysis observed, which was consistent with previous observations (Figure 4.2.11). However, multiple repeats continued to show significant variation using serum diluted to 50% probably as a result in variation in the level of active complement contained within each dilution (Figure 4.2.14C).

Importantly, the cytolytic activity of undiluted serum was 2-fold greater than that for 50%, producing fluorescence intensities similar to those observed from total lysis (Figure 4.2.14A). With the assumption that 0.1% triton-X 100 is providing total cell lysis, when Calcein-release is expressed as a percentage of the population, 100% serum falls short of total lysis, by 6-8% (Figure 4.2.14B). However, this may simply

be the upper-limit of the assay or representative of the number of low or non-α-gal expressing cells (Figure 3.2.12).

The use of undiluted, freshly acquired or freshly frozen, human serum combined with an improved assay protocol based on that described by Iwanowicz *et al.,* (2004) using 40μM Calcein.AM instead of 2μM, for 2 hours, has resulted in an extremely robust method for the detection of complement-mediated lysis of PDFF cells, using the presence of the α-gal epitope and natural human anti-α-gal antibodies. The next objective was to evaluate the use of the optimised assay to detect complement-mediated lysis of the transgenic M2 and F11 cell lines when exposed to human serum.

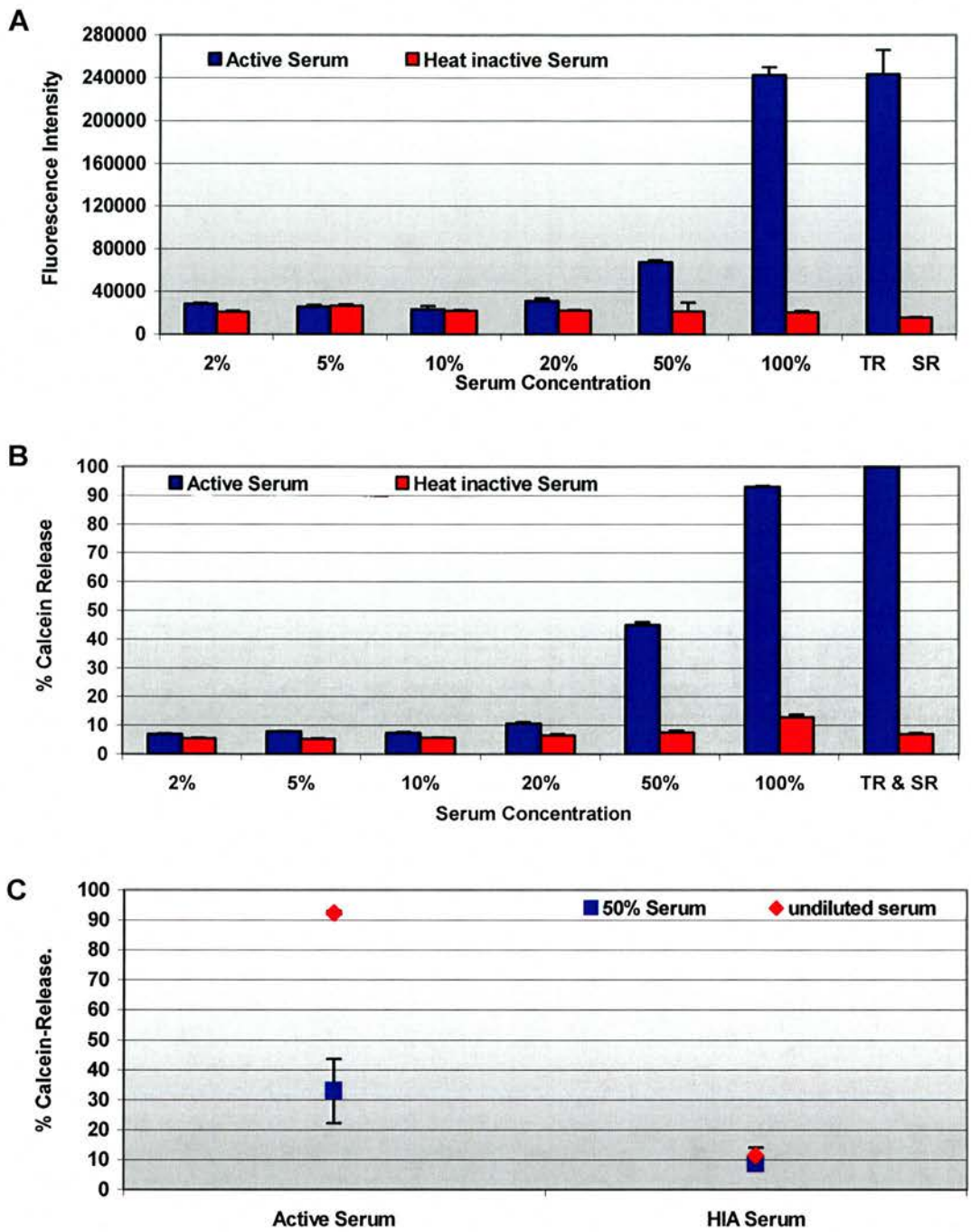


Figure 4.2.14: Undiluted serum significantly increases cytolytic activity, but reduced inter-experimental variation, without increasing non-specific cell killing. A] Lysis with undiluted serum releases similar amounts of Calcein as 0.1% triton-X 100. B] Undiluted serum doubles the level of specific PDFF lysis compared to using serum diluted to 50%. C] Undiluted serum (◆) significantly reduces inter-experimental variation compared to serum diluted to 50% (■) data from three independent replicates. Active serum is represented in blue, heat inactivated (HIA) serum in red. Error bars represent the standard deviation of the individual observations.

4.2.8 Complement-Mediated Cytotoxicity is Specific to Cells which Express the α-gal Epitope with High Efficiency in the Presence of Human Serum.

With an optimised assay, an assessment of complement-mediated cell lysis, specific to the α-gal epitope was performed. PDFF, M2, F11 and H9 cells (10^5) were loaded with 40μM Calcein.AM for 2 hours diluted in 50μl of appropriate culture medium. Cells were then incubated with 100μl of undiluted active or heat inactivated human serum, or 100μl of appropriate culture medium, for an hour at 37°C in a humidified incubator containing 5% CO_2. The culture supernatant was then assessed for the presence of released Calcein as previously described.

As expected, PDFF cells were lysed in a specific manner resulting in excess of 80% Calcein-release. Interestingly, the same level of specificity was also observed for the first time in the transgenic M2-ES cell line, and as would be expected, the reduced level of α-gal expression on the F11 cell line resulted in an increased number of cells surviving the complement-mediated lysis (Figure 4.2.15A). Furthermore, there was minimal lysis of the wild type ES cells (H9), just 8%, well below the accepted level for spontaneous background release (Figure 4.2.15A).

Furthermore, the optimised assay conditions have provided a reproducible assay not only for PDFF cells, but also for the human ES cells, clearly indicating that human ES are susceptible to complement mediated lysis, but that in this instance it is restricted to the presence of the α-gal epitope (Figure 4.2.15B).

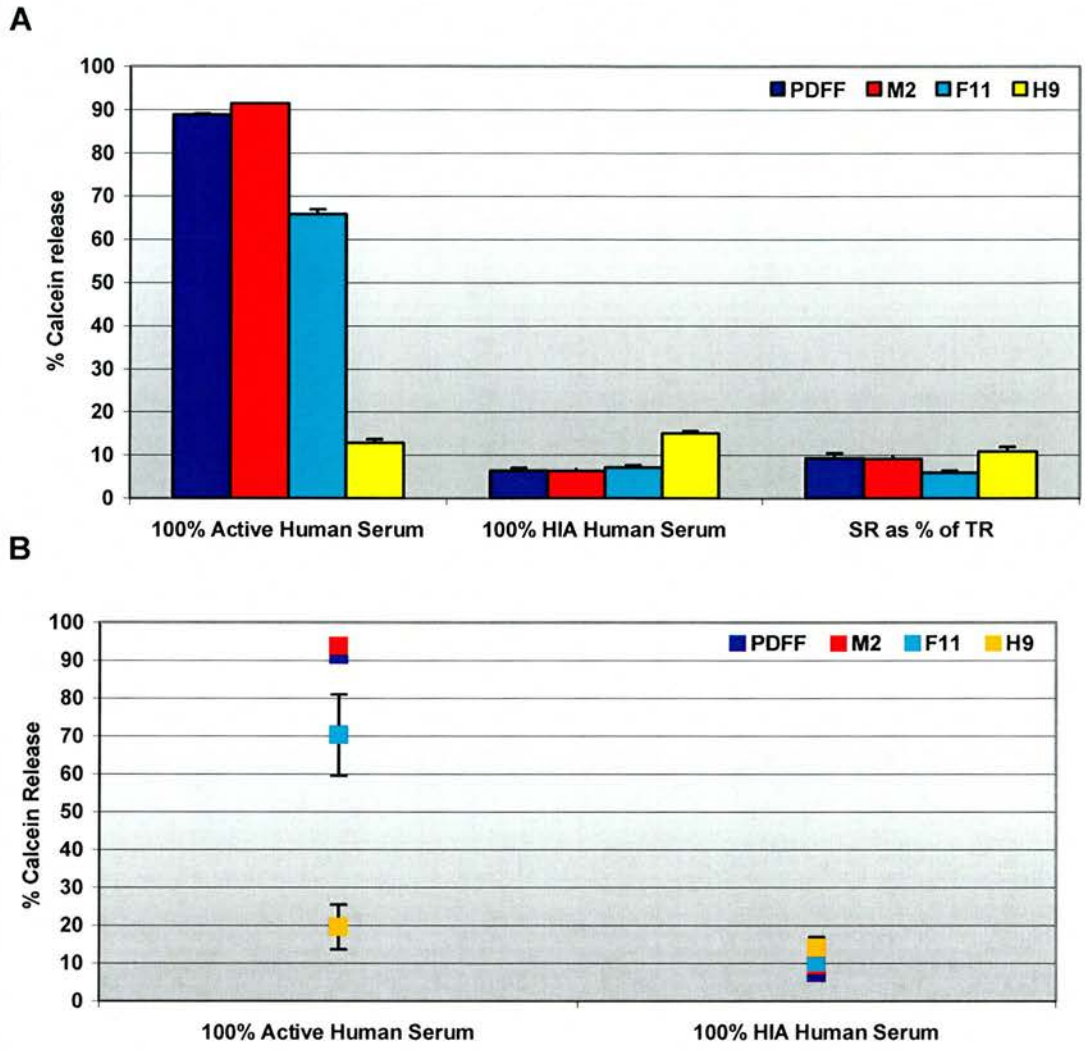


Figure 4.2.15: M2 cells are as susceptible to complement-mediated lysis as PDFFs. A] Both PDFF (blue) and M2 (red), released ~90% of their loaded Calcein in response to human serum. F11 (sky blue) were less susceptible to complement attack, releasing 66% Calcein. Non-specific Calcein-release from H9 (yellow) was 13%, significantly less than the accepted background level. B] Using the newly defined assay conditions, the level of complement-lysis has been shown to be reproducible for both PDFF (blue) and M2 (red). The F11 clone (sky blue), however, is more variable than the M2 clone over three independent replicates. Error bars represent the standard deviation of the individual observations.

4.2.9 Blocking Complement-Regulatory Proteins, CD55 and CD59, with Monoclonal Antibodies has no Effect on the Susceptibility of Human ES Cells to Complement-Mediated Lysis.

The development of a reliable and reproducible assay provided an opportunity to investigate the biological function of expressing elevated levels of complement inhibitory proteins. To do this wild type H9-ES cells were coated with non-complement fixing blocking antibodies, mouse IgG_1, MEM-43 and HD3, anti-CD59 and anti-CD55 respectively, (a kind gift from Prof. P. Morgan, University of Wales) and exposed to complement. To activate complement-mediated lysis, exogenous antibody to a cell surface epitope (IgM to TRA-1-81) was diluted in active human serum. TRA-1-81 is an endogenous cell surface marker, used as part of a panel to characterise undifferentiated human ES cells (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998). It is expressed on over 90% of undifferentiated human ES cells with a mean fluorescence intensity of 3,820 (Figure 7.2.2 and Table 7.2.1). To control against non-specific complement activation as a result of bound blocking antibody, H9-ES cells were incubated in the absence of the Tra-1-81 activation antibody (Figure 4.2.16).

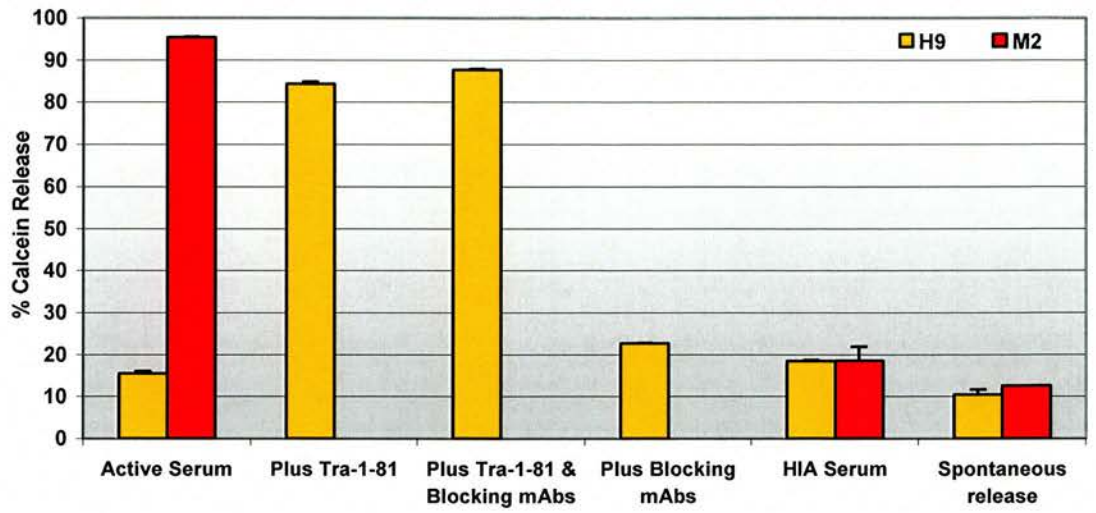


Figure 4.2.16: Blocking of complement inhibitors with monoclonal antibodies does not significantly increase the level of complement-mediated lysis. H9 cells (yellow) were incubated with active serum alone, plus activating Tra-1-81 antibody, plus blocking antibodies or plus blocking antibodies and Tra-1-81, heat inactivated (HIA) serum and culture medium. M2 cells (red) were incubated with active and HIA serum to compare the efficiency of the complement mediated lysis. Data represents the mean from duplicate experiments and the error bars represent the standard deviation of the individual observations.

The addition of exogenous Tra-1-81 IgM produced an effective complement-mediated lysis response, leading to 84% Calcein-release. Surprising, this was some what lower that the lysis response to α-gal antibodies, which are mainly of IgG isotype (95% Calcein-release; Figure 4.2.16), suggesting that there was the need from optimisation. Pre-coating H9 cells with blocking antibodies to complement inhibitors, before incubation with Tra-1-81 and serum, slightly increased the level of lysis (88% Calcein-release; Figure 4.2.16). However, there was also an increase in the level of background lysis (23%) over that observed for heat inactivated (HIA) or active serum alone (18%; Figure 4.2.16). The working hypothesis had been that binding antibody to the two highly expressed complement inhibitors, CD55 and

CD59, would remove any protective capacity that they had towards undifferentiated human ES cells. Consequently, it was hypothesised that there would be an increase in the level of cell lysis (Calcein-release). The data presented here suggested one of two things, either that the increased expression of complement inhibitor proteins was insufficient to protect human ES cells from a specific complement mediated attack, or that in both examples, natural α-gal and exogenous IgM to TRA-1-81, saturation of the system occurred, providing no opportunity for the inhibitors to function.

Perhaps a more informative experiment would have been to expose human ES cells to sub-optimal levels of anti-α-gal or Tra-1-81, i.e. to dilute the serum to levels which are on the lower limit of being able to cause lysis, between 20 and 50% (Figure 4.2.14) and then asking the question: if human ES cells have increased complement inhibitor activity then would the level of lysis increase if this protective action was lost (bound by antibody)?

4.3 Discussion

4.3.1 Optimisation of the Calcein-release Assay for Human ES Cells

It is clear from the results in this chapter that different cell types vary in their ability to take up and retain the Calcein.AM label (Figure 4.2.9). The conditions reported by Spiller (2000) using 2μM Calcein.AM to load 10^7 cells/ml resulted in very high levels of intra- and inter- experimental variation. When increased levels of Calcein.AM were investigated, it transpired that at 2μM Calcein.AM there was insufficient discrimination between spontaneous release and specific release as a result of either detergent or serum lysis. Furthermore, Calcein saturation was not achieved in any cell type at 80μM, a 5-fold increase in the level of Calcein.AM, thus when complement-mediated lysis had occurred, using the method of Spiller (2000) the assay was not sensitive enough to detect it. Additionally, the technique reported by Spiller, (2000) of using 250μl of serum or detergent to lyse the cells, and then collecting all of this to measure the level of Calcein-release was difficult to achieve inside laminar-flow hoods because of the small size of the surviving cell pellet and the angle of the glass, adding to experimental variation through operator error. The technique reported by Iwanowicz *et al.,* (2004), significantly reduced this operator error, since it did not require 100% of the lysis supernatant to calculate the percentage Calcein-release, helping to prevent contamination of supernatant fluorescence with cell pellet fluorescence from viable cells. Consequently, a switch to this protocol provided more consistent results and lower standard deviations within each experimental run (Figure 4.2.1 compared with Figure 4.2.10).

The issue of inter-experimental variability of the Calcein-release assay was not, however, confined to the mechanics of the assay. Sub-optimal use of serum, both source and concentration, further exacerbated the problem. The cytolytic activity of fresh and freshly frozen serum was some 30% higher than that achieved with commercial serum. The reason for this was probably the initial storage (-20°C) and transport thawing of commercial serum, which resulted in degradation of complement proteins. Storing commercial serum at -80°C upon arrival prevented further degradation (Figure 4.2.13), confirming the observation that complement proteins are unstable when stored above -80°C (Spiller B. Pers. Com.) and providing an explanation for the low level of complement-mediated lysis observed in the early experiments and some of the inter-assay variation (Figure 4.2.12).

A titration of the serum concentration found that using serum diluted to 50%, as reported by Huang *et al.,* (2001) for the lysis of NIH/3T3 cells, resulted in 40-50% specific Calcein-release, while using undiluted serum increased the specific lysis to 80-90%, without significantly increasing the level of non-specific cell lysis. Furthermore, serum dilution was adding to inter experimental variation, and therefore, it was not surprising that the use of undiluted serum significantly decreased inter-experimental variation (Figure 4.2.14).

With the Calcein-release assay optimised for the detection of complement-mediated lysis in PDFF cells, only a small adjustment in the concentration of Calcein.AM (40μM instead of 20μM - Figure 4.2.9) was necessary to provide sensitive assay conditions for detection of lysis in human ES cells. Importantly, H9 wild type ES

cells were insensitive to complement-mediated lysis directed against the α-gal epitope and were only sensitive to lysis when antibody against the endogenous cell surface marker Tra-1-81 was added to the serum, showing specificity of the assay.

Transgenic M2 and F11 human ES cell clonal lines on the other hand were both successfully lysed as a result of expressing the α-gal epitope on their cell surface. As predicted, the F11 cell line was less efficiently lysed than the M2 (Figure 4.2.15) due to its lower level of α-gal expression and increased variegation (see section 3.2.2.2). However, in contrast to the expression data reported in Chapter 3, (Figure 3.2.12) the M2 cell line was lysed as efficiently as the endogenously expressing PDFF cells (Figure 4.2.15), despite apparently expressing less α-gal epitopes and binding lower levels of anti-α-gal (Figure 3.2.14). A possible explanation for this result is that the M2 cells, which have a smaller cell surface area than the PDFF cells, bound fewer anti-α-gal antibodies per cell, (mean fluorescence intensity of 447.48 compared to 913.34 for PDFF's) but it is likely that the antibodies that did bind were in closer proximity to each other. Initiation of the classical complement cascade requires the binding of 2 molecules of IgG antibody to occur in close proximity, within 30-40nm of each other, to then be able to bind to the first complement protein C1q (Goldsby *et al.,* 2000). M2 cells were capable of binding very similar levels of both *BS-IB$_4$* and anti-human Ig, to PDFF cells, despite their reduced size. It is therefore hypothesised that since the majority of anti-α-gal is IgG, that the reduced cell size of human ES cells meant that although there where fewer α-gal epitopes than where present on the larger PDFF cells, those that were there were closer together increasing the chance of 2 anti-α-gal IgG antibodies binding in close enough proximity to initiate lysis. This

would explain the observed similarities in the efficiency of complement-mediated lysis of both PDFF and M2 cell lines and would also account for the differences α-gal expression as observed by a higher fluorescence intensity by flow cytometry following FITC conjugated *BS-IB$_4$* staining, and in increased levels of FITC conjugated anti-human Ig binding.

4.3.2 Expression of Complement Inhibitory Proteins on Human ES Cells

Complement-mediated lysis directed against an endogenous cell surface marker of human ES cells, TRA-1-81, using the addition of exogenous antibody, was successfully initiated, demonstrating that supra-optimal levels of exogenous antibody can supersede any protective effects of having increased complement inhibitor proteins. Additionally, these data suggested the possibilities of using this technique with vaccination strategies against foreign epitopes.

Complement inhibitor proteins, are essentially involved with preventing spontaneous activation of the complement cascade, caused either as a result of non-specific binding of self-antibodies or through the constant "ticking-over" of the key complement protein C3 (Atkinson & Farries, 1987). However, the frequency at which this background binding takes place is likely to be orders of magnitude lower than the specific binding of both natural levels of anti-α-gal antibody and supra-optimal levels of exogenous Tra-1-81 antibody, leading to saturation of the system and over-whelming the ability of complement inhibitor proteins to act against complement initiation.

In a recent report, Harrower et al (2004) produced transgenic pigs which over-expressed human complement inhibitor proteins, CD59 (MIRL) or CD46 (MCP), in an attempt to overcome the effects of hyperacute rejection. However, when exposed to active human serum, embryonic neural tissue from pigs transgenic for either human CD59 or human CD46 was not protected from the effects of activated complement (Harrower *et al.,* 2004), confirming the hypothesis that elevated levels of complement inhibitory proteins are not capable of protecting against a directed complement attack.

Complement regulatory proteins, particularly DAF, are highly expressed on trophoblast cells from 6 weeks of gestation (Holmes *et al.,* 1990). They are thought to be involved in the protection of the foetus from maternal complement. Loss of these proteins at the foeto-maternal interface has been shown to be associated with foetal loss in mice and has been associated with spontaneous abortion (Xu *et al.,* 2000; Holmes *et al.,* 1990, 1992). It comes as little surprise therefore, that these proteins are highly expressed in human ES cells to the same extent as they are expressed in the human embryonic kidney cell line (HEK 293) (Figure 4.2.7). However, with the development of a reliable Calcein-release assay to detect complement-mediated lysis in human ES cells, it is clear that the increase in complement inhibitors was insufficient to protect against a directed attack.

4.4 Conclusion

The Calcein-release assay, once optimised, has proven to be a very reliable method for the detection of complement-mediated lysis. Using these optimised conditions human ES cells, expressing the α1,3Gal transgene, have been repeatedly shown to be highly susceptible to complement-mediated lysis, to the same extent as α1,3Gal endogenously expressing PDFF cells, in the case of the M2 human ES cell line. Furthermore, the addition of supra-optimal levels of exogenous antibody to an endogenous cell surface epitope was also capable of eliciting a complement-mediated attack, providing the opportunity to develop this technique in conjunction with vaccination strategies.

CHAPTER 5 COMPLEMENT-MEDIATED LYSIS AS A TOOL FOR THE SELECTIVE ABLATION OF UNDIFFERENTIATED HUMAN ES CELLS.

5.1 Introduction

The persistence of undifferentiated human ES cells within a potentially therapeutic population poses a serious risk of tumorigenicity to the graft recipient. Critical to the success of the selective ablation strategy presented in Chapter 4, is the absence of α-gal epitopes on differentiated derivatives of the M2 human ES cell line. The expression of the α1,3Gal transgene from the hTERT promoter should provide a method for identifying such cells, through specific expression of the α-gal epitope on undifferentiated human ES cells, which can then be removed or ablated. As

discussed in Chapter 1, Tzukerman *et al,* (2000) have shown that there is a time-dependent decline of telomerase activity, associated with a decline in promoter activity, upon induction of differentiation in human ES cells. The authors observed disappearance of hTERT activity after 14 days of differentiation (Tzukerman *et al,* 2000). This chapter will demonstrate the specificity of complement-mediated lysis, as an elimination strategy, to distinguish between cells that do and do not express the α-gal epitope. It will assess the expression of α1,3Gal on differentiated derivatives of M2 cells, and determine their sensitivity to complement-mediated attack.

5.1.1 Chapter 5 Aims

1. To qualify the level of complement-mediated lysis indicated by the Calcein-release assay, to determine the level and nature of surviving cells.

2. To determine whether minority populations of undifferentiated M2 cells can be selectively eliminated from a population of α-gal negative cells.

3. To differentiate M2 ES-cells and assess the expression of α-gal on differentiated derivative.

4. To determine the level of susceptibility in differentiated derivatives of M2 to complement-mediated lysis.

5.2 Results

5.2.1 Complement-Mediated Lysis is Underestimated Using the Calcein-Release Assay.

In an attempt to qualify the Calcein-release assay as a method of determining complement-mediated lysis, unlabelled PDFF, M2 and H9 cells were exposed to complement (10^5 cells in 100µl of undiluted active and heat inactivated serum) for 1 hour at 37°C. Surviving cells were then pelleted at 200g for 5 minutes and resuspended in 100µl of culture medium. In duplicate wells of a 12-welled plate (NUNC), coated with an appropriate extra-cellular matrix, 50µl of cell suspension (maximum of $5x10^4$) were re-plated and cultured overnight at 37°C in a humidified incubator plus 5% CO_2.

Twenty-four hours post treatment the survival of PDFFs, M2 and H9 cells was assessed by 5% Giemsa staining (Figure 5.2.1). Surprisingly, the lysis of both PDFF and M2 was more acute than expected from the results of the Calcein-release assay reported in Chapter 4. Figure 5.2.1 shows representative images, from three independent experiments, indicating the almost complete elimination of α-gal expressing cells as a result of incubation with active human serum (Figure 5.2.1 a and c), while α-gal negative cells were unaffected by incubation in active serum (Figure 5.2.1b).

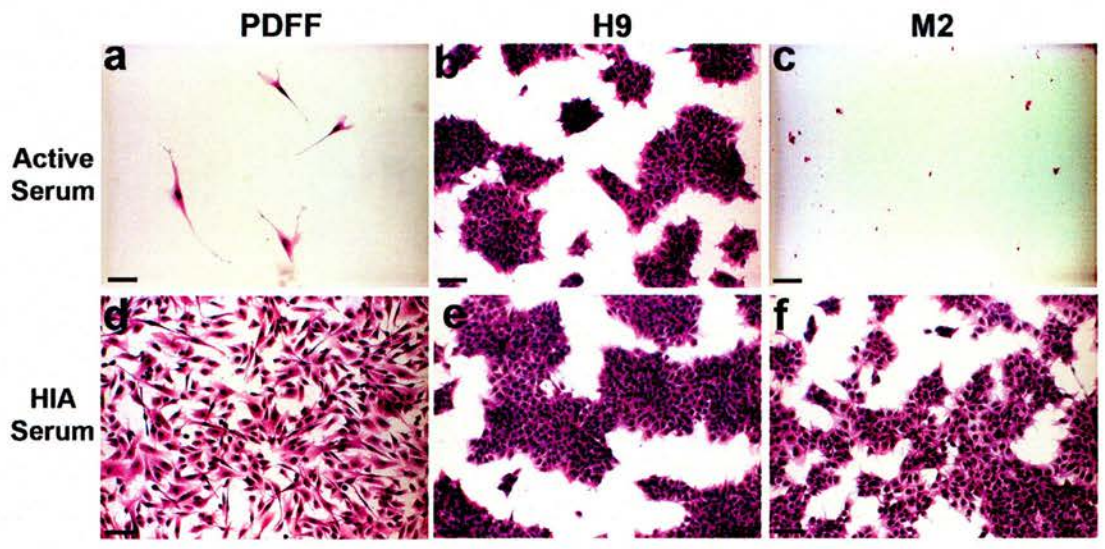


Figure 5.2.1: Giemsa staining of cells following a 1-hour incubation in active (upper panel) and heat inactivated (lower panel) human serum, shows specificity of serum lysis to the presence of the α-gal epitope. H9 (b and e) cells are unaffected by incubation with active (b) while PDFF (a and d) and M2 (c and f) cells are highly sensitive to serum lysis with active (a and c) human serum but not heat inactivated (d and f) human serum. Survival of cells assessed 24 hours post lysis with 5% Giemsa staining, scale bar represents 100um.

In parallel experiments, duplicate wells containing cells exposed to active and heat inactivated human serum were set up to determine if lysis, while specific, was also complete. These wells were allowed to grow under normal culture conditions following treatment to induce lysis, for 7-10 days. Interestingly, in all cases, small ES colonies did appear after a 10-day period from the M2 population treated with active human serum (Figure 5.2.2). Cell survival suggests that a single one-hour incubation was not sufficient to kill all α-gal expressing cells within a large population, possibly due the presence of small cell aggregates within the cell suspension. The presences of cell aggregates would prevent cells in the centre of the aggregate from being exposed to active complement and consequently when the cells

were washed, removing the dead cells, this would allow the viable cells from within the aggregates to adhere and proliferate. Alternatively, it is possible that there was simply insufficient complement within the serum to lyse the whole population within an hour and that by either increasing the amount of serum or the duration of exposure would result in total cell lysis.

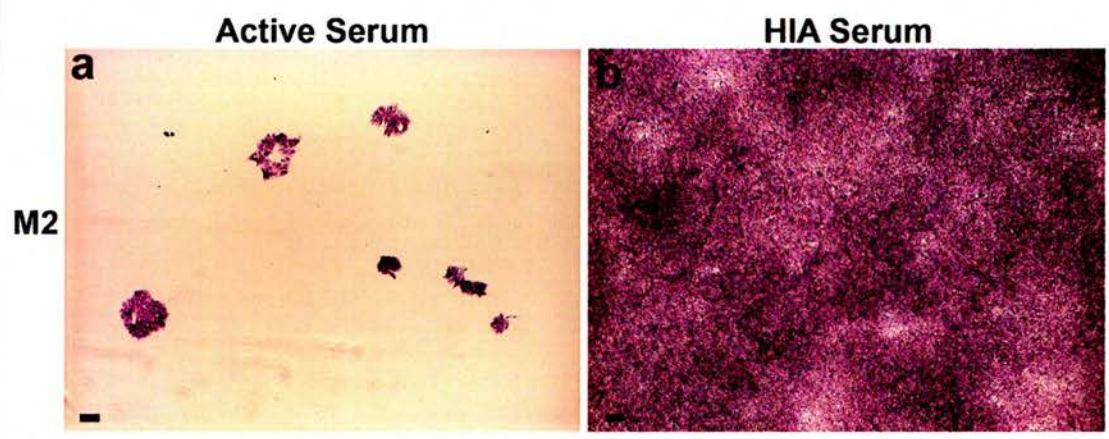


Figure 5.2.2: Small colonies of human ES cells do occur 7-10 days after incubation with active human serum (a), suggesting that a single 1-hour incubation is not sufficient to kill all α-gal expressing cells in a large population. Panel b shows the level of growth after 7-10days of cells incubated with heat inactivated human serum. Scale bars represent 100μm.

5.2.2 Surviving Cells Remain BS-IB$_4$ Positive, Suggesting α-Gal Positive Cells had Avoided Cell Lysis.

Another hypothesis for the persistence of M2 cells following complement attack was that the remaining cells did not survive complement attack; they simply avoided death because they had no α-gal epitope to initiate a complement-mediated attack. In Chapter 3 the level of α-gal expression on the cell surface of the M2 cell line was

reported as 95% (Figure 3.2.12), suggesting the presence of α-gal negative cells within the population, which could account for the persistence of cells after complement attack.

Consequently, surviving M2 colonies were assessed for expression of α-gal by staining with FITC conjugated *BS-IB$_4$* lectin. Without exception, all surviving M2-ES cell colonies stained positive for α-gal (Figure 5.2.3), suggesting that either these cells had escaped complement-mediated lysis despite the presence of the α-gal epitope, or that as a result of variegation within the clone, these cells did not express the epitope at the time of complement attack, but up-regulated it during the subsequent *in vitro* culture.

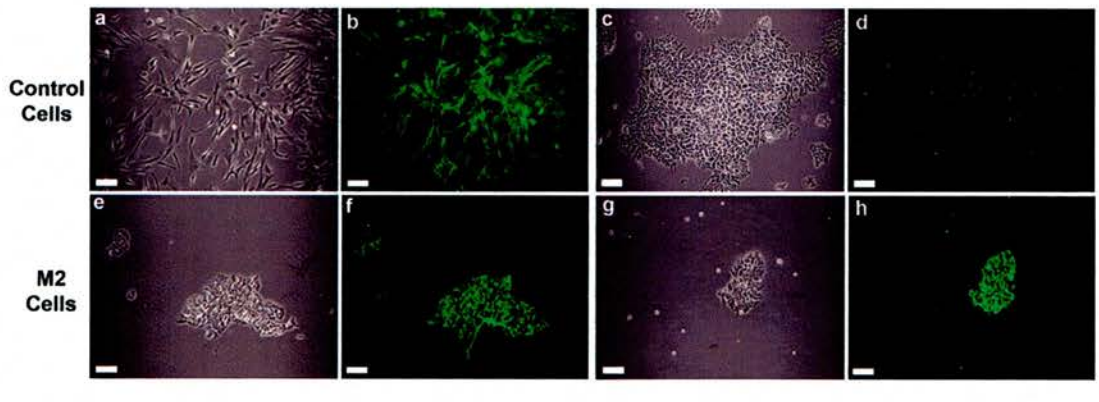


Figure 5.2.3: Surviving PDFF and M2 cells remain positive for α-gal by *BS-IB$_4$* staining. PDFF (a and b) and surviving M2 (e - h) cells were cultured for 10 days post-incubation with active serum and then staining with *BS-IB$_4$* for presence of the α-gal epitope. H9 (c and d) surviving cells cultured for 10 days at low density (10^5/ml) did not stain positive with *BS-IB$_4$*. Scale bars represent 100μm.

5.2.3 M2 ES-Cells can be Removed as a Minority Group from Populations of α-Gal Negative HEK 293 Cells by Complement-Mediated Lysis.

The data for elimination of α-gal positive cells by complement-mediated lysis reported thus far have concentrated on eliminating a whole population of α-gal expressing cells. However, in a clinical setting, it is likely that the contamination of therapeutic populations with undifferentiated human ES cells will be low. Therefore, in an attempt to mimic this clinical setting, α-gal positive M2 cells, were mixed in serial dilutions with α-gal negative HEK 293 (Figure 5.2.4) at ratios of 50, 25, 12.5, 6.3, 3.15, 1.6 and 0.8% and then exposed to complement-mediated lysis. To ensure specific lysis, pure populations of both M2 cells and HEK 293 cells were also included, as were a H9 negative control and PDFF positive control (Figure 5.2.1).

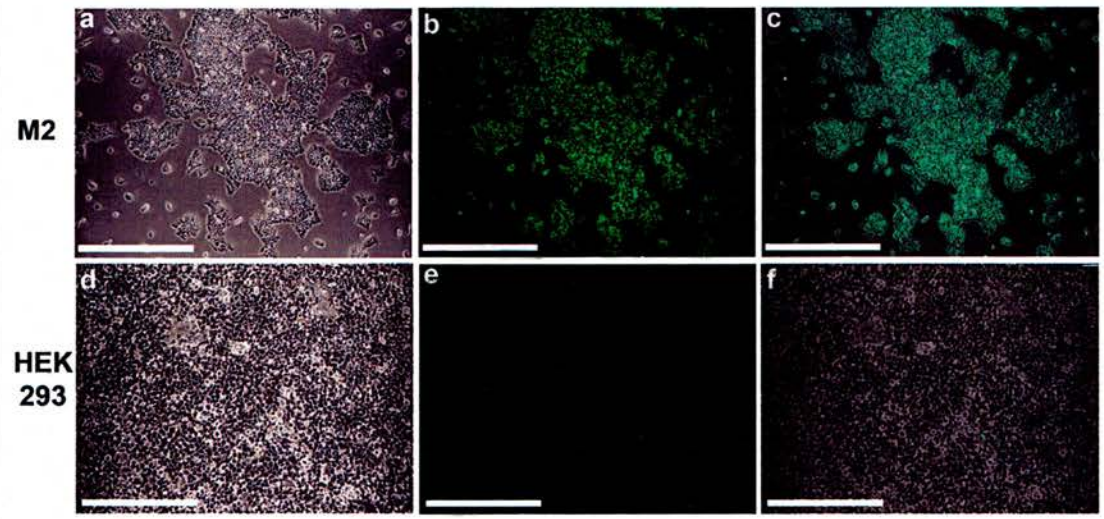


Figure 5.2.4: *BS IB$_4$* staining of transgenic M2 cells, positive for α-gal expression (a-c) and HEK 293 cells negative for α-gal (d-f). Images a and d show phase contrast microscopy, b and e show FITC fluorescence microscopy and images c and f overlay the two. Scale bars represent 100μm.

Cells were harvested, counted and resuspended at $1x10^5$/ml in culture medium. Population mixing was then carried out to give a final concentration of 10^5/ml of mixed cells. The mixed populations were then pelleted at 200g for 5 minutes, and finally resuspended in 100µl of undiluted active or heat inactivated human serum. After a 1-hour incubation in the presence of serum, the populations were collected, following centrifugation at 200g for 5 minutes and resuspended in 100µl of culture medium. 50µl of cell suspension (maximum of $5x10^4$ cells) per well were re-plated into duplicate wells of a 12-welled plate (NUNC), coated with an appropriate extra-cellular matrix, and cultured overnight at 37°C in a humidified incubator plus 5% CO_2. To confirm the level of population mixing, cells exposed to heat inactivated serum were stained 24 hours post-incubation for the ES specific marker Oct-4, (Figure 5.2.5). Oct-4 staining in Figure 5.2.5 clearly shows a gradual decline in the number of undifferentiated ES cells within the HEK 293 cell population, indicating that at each dilution, Oct-4 positive ES cells were present prior to treatment with human serum (Figure 5.2.5).

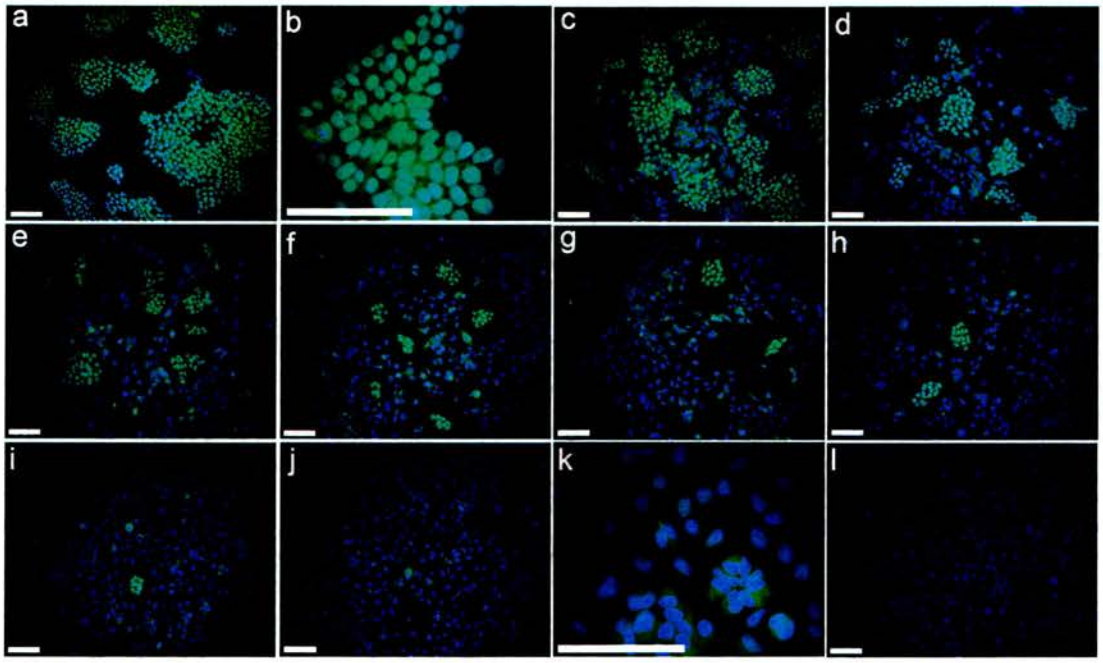


Figure 5.2.5: HEK 293 and M2 mixed populations 24 hours post-incubation with heat inactivated human serum. M2 ES cells mixed with HEK 293 cells at ratios of 100% (a & b at x10 and x40 respectively), 50%, 25, 12.5, 6.3, 3.15, 1.6 and 0.8% (c to I respectively) and 0% (j & k at x10 and x40 respectively). Oct-4 staining (FITC) stains the nuclei of undifferentiated human ES cells, background cytoplasmic staining can be seen in HEK 293 cells (k), however, their nuclei remain blue with DAPI. Image l shows incubation of that undifferentiated human ES cells with the secondary FITC antibody and DAPI, clearly demonstrating that Oct-4 staining was specific. Scale bars represent 100um.

After plating, all mixed populations were placed in selective medium (1mg/ml G418, as determined by a HEK 293 and M2 kill curve, data not shown) to remove the HEK 293 cells while allowing the proliferation of the transgenic M2 cells that had escaped lysis. Populations were cultured in this way until total cell death had occurred in the pure HEK 293 population, 7-10 days at 1mg/ml G418 (data not shown), signifying that any remaining G418 resistant cells in the mixed populations were probably of M2 origin. Cell survival was assessed by colony counting from duplicate wells in

three independent experiments (Table 5.2.1) based on morphology and by 5% Giemsa staining (Figure 5.2.6).

Percentage M2 Population	Number of surviving ES colonies						Frequency
	Replicate 1		Replicate 2		Replicate 3		
	A	HIA	A	HIA	A	HIA	
100.00%	34	>500	11	>500	12	>500	<3.80%
50.00%	6	>500	8	>500	25	>500	<2.60%
25.00%	1	>500	6	>500	13	>500	<1.33%
12.50%	0	>500	0	>500	4	>500	<0.27%
6.30%	0	>500	0	>500	3	>500	<0.20%
3.15%	1	>500	0	>500	0	>500	<0.07%
1.60%	0	480	0	435	1	>500	<0.07%
0.80%	0	242	0	216	0	466	0.00%

Table 5.2.1: The frequency of proliferating ES colonies post-lysis, after 7-10 days in selective culture. The number of colonies surviving complement-mediated lysis in each replicate are denoted A, for active serum, columns headed HIA represent the number of colonies expected for the proportion of ES cells included in the mixed population, i.e. the same population mixes incubated with heat-inactivated serum. Counts were based on morphology using phase contrast microscopy at x10 magnification, colonies in heat-inactivated serum were too confluent to count in most cases resulting in an under estimation of colony number and consequently, the reported frequencies are an over estimation.

Table 5.2.1 clearly indicates that as the ratio between sera to cells increases, there is a significant reduction in the number of surviving ES cells. In populations containing less than 25% M2 cells, only a single colony from duplicate wells, from 3 independent experiments, was ever observed, and no colonies were ever observed when the proportion of M2 cells was 0.8% (Table 5.2.1 and Figure 5.2.6). However, as previously observed, at higher concentrations of M2 cells, colonies were occasionally observed, albeit at low frequency. It was estimated that a confluent well of a 12-well plate contains some $3.3x10^5$ undifferentiated human ES cells, if this was

taken to be the case in the above experiments, the frequency of surviving ES cells in 100 and 50% population mixes would have been in the region of 0.0057% and 0.0033% respectively. Perhaps a criticism of this result is that a total cell count, instead of a colony count, would have provided a more accurate frequency of cells surviving complement mediated lysis. Nevertheless, these results further indicate that at high cell concentrations, a single 1-hour incubation in active human serum kills most but not all α-gal expressing cells, probably as a result of cell aggregating together when at high densities (Figure 5.2.6).

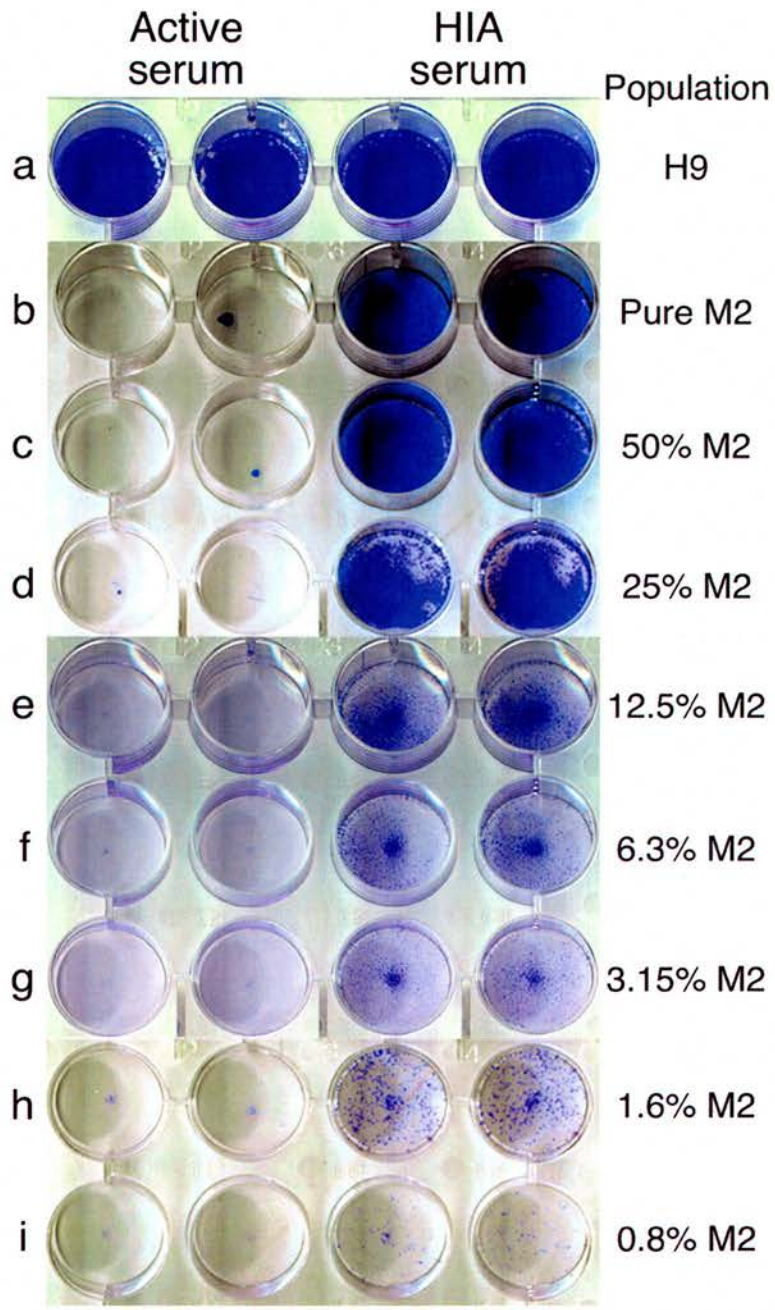


Figure 5.2.6: Visual representation of the number of proliferating M2 ES cell colonies from a mixed population surviving exposure to complement, stained with 5% Giemsa. Mixed HEK 293 and M2 populations (containing pure, 50, 25, 12.5, 6.3, 3.15, 1.6, and 0.8% M2 cells, images b-i respectively) were cultured in 12-well plates in the presence of 1mg/ml G418 for 7 days following incubation with undiluted active and heat inactivated (HIA) human serum. Image a, represents $5x10^4$ H9 cells cultured for the same 7 day period without G418 selection.

5.2.4 Expression of α-Gal is Down-Regulated Following Differentiation, but is Persistent in a Small Sub-Population

As previously mentioned, for this strategy to be useful therapeutically, down-regulation of the α-gal epitope is essential in differentiated populations. Characterisation of α-gal down-regulation will be described fully in Chapter 7 (Figure 7.2.3). Shown here are fluorescence microscopy images demonstrating moderate down regulation of α-gal expression following ten days in basic differentiation medium (KO-DMEM supplemented with 10% FBS (v/v), 0.1mM NEAA, 2mM L-glutamine and 0.1mM β-mercaptoethanol, Figure 5.2.7 (images a-c)) and for comparison the expression level of TRA-1-81, a reported marker of ES and EC cells, which is down-regulated on differentiation, at the same time point (Figure 5.2.7 (images d-f)).

As shown in Figure 5.2.7, M2 cells had begun to down-regulate TRA-1-81, indicating that the cells were differentiating. The corresponding reduction in *BS-IB$_4$* staining supports the assumption that the α1,3Gal transgene was being appropriately regulated. Visually, it appeared that M2 cells had down-regulated the expression of α-gal more than TRA-1-81, perhaps indicating tighter regulation of the hTERT promoter. Furthermore, those cells which had retained α-gal expression either had an ES phenotype or were located near to nests of ES like cells, suggesting a transient persistence of the epitope, following down-regulation of transcription (Figure 5.2.7 (images a-c)).

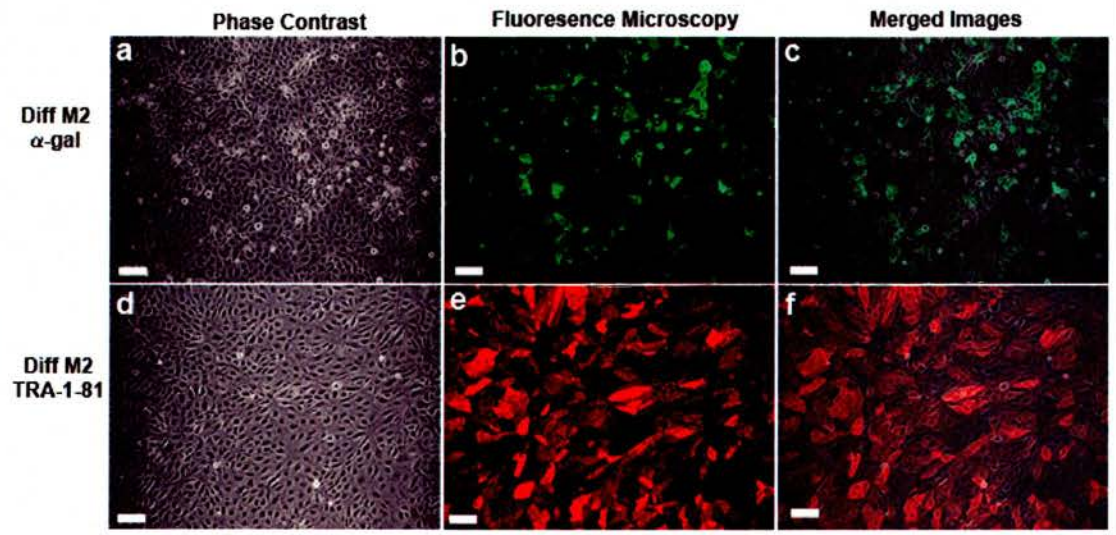


Figure 5.2.7: M2 cells differentiated for 10 days begin to down regulate α-gal (FITC-green) expression in a similar manner to TRA-1-81 (R-PE-red), a reported marker of ES and EC cells. M2 cells, differentiated in basic differentiation medium for 10 days, were assessed for the presence of α-gal by *BS-IB$_4$* staining and TRA-1-81. Images a and d show that the morphologies of the differentiated cells are similar by phase contrast micrscopy. The level of continued α-gal (*BS-IB$_4$* staining (FITC) b and overlay c), and TRA-1-81 expression (R-PE staining e and overlay f) were determined by fluoresence microscopy showing down-regulation of both TRA-1-81 and α-gal after 10 days of differentiation. Scale bars represent 100μm.

5.2.5 Differentiated M2 Cells Become Insensitive to Complement-Mediated Attack Initiated by α-Gal Epitopes, with Increased Time in Differentiation Medium.

To determine that a loss of *BS-IB$_4$* staining corresponded to a loss of the α-gal epitope and consequently protection from complement-mediated lysis, differentiated M2 cells were exposed to active human serum at increasing time points following induction of differentiation.

Differentiation of M2 cells, cultured as monolayers, was induced for 7, 14 and 21 days by replacing conditioned medium and hbFGF with basic differentiation medium containing 10% FBS. Undifferentiated M2 cells and PDFF cells were used as positive controls, and equally differentiated and undifferentiated H9 wild type cells as negative controls. Cells were loaded with Calcein as previously described (10^5 cells with 40μM for 2 hours) and then exposed to active and heat inactivated human serum for one hour at 37°C.

The level of specific complement-mediated lysis, percentage Calcein-release minus spontaneous release following incubation with heat inactive serum, significantly decreased as the differentiation time increased: 85, 62, 25 and 8% at 0, 7, 14 and 21 days respectively (Figure 5.2.8). This suggested that with increasing time in differentiation M2 cells become less susceptible to α-gal induced complement-mediated lysis.

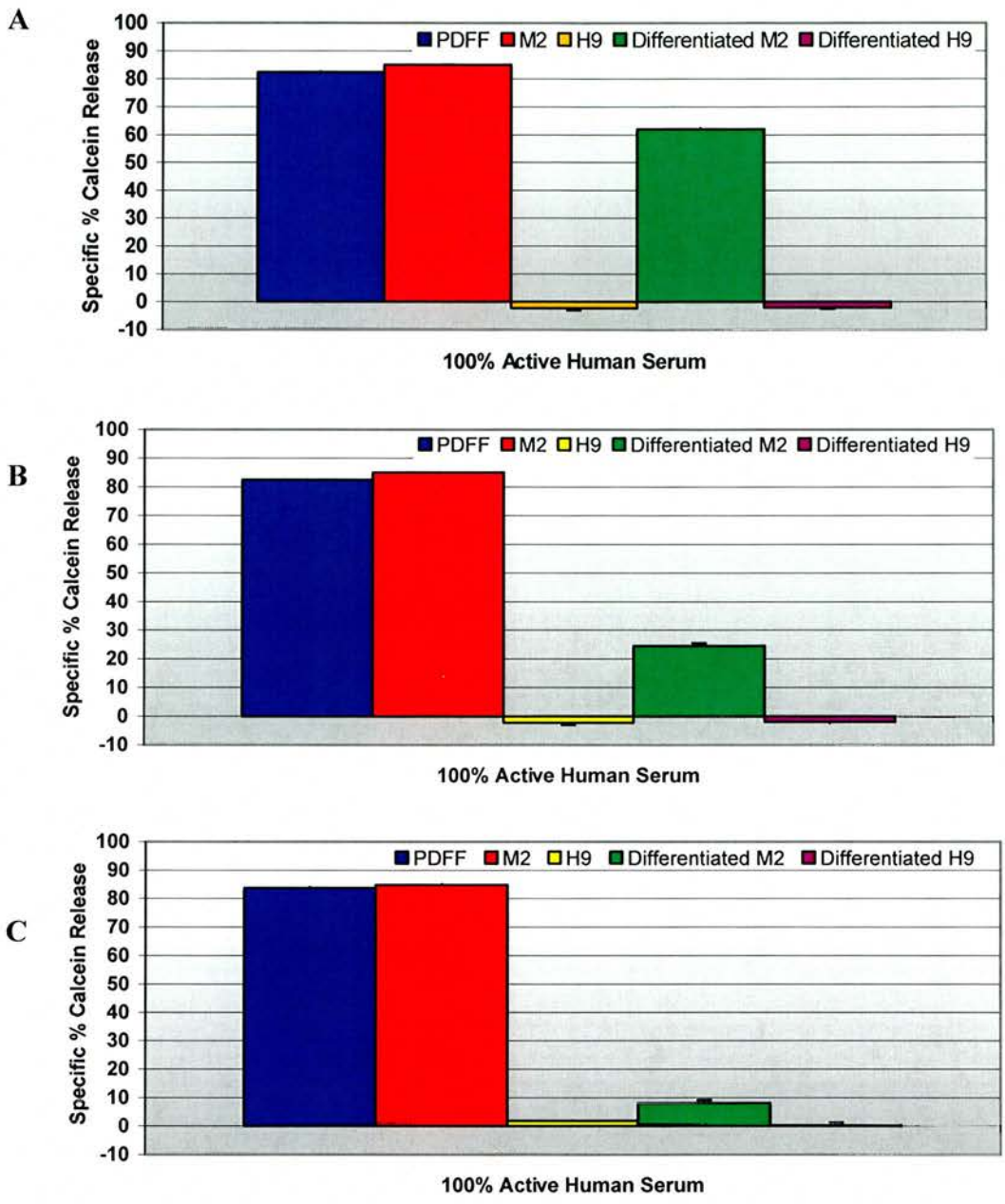


Figure 5.2.8: Differentiated M2 cells become less susceptible to α-gal induced complement-mediated lysis with increasing time in differentiation. M2 (red) and H9 (yellow) cells were differentiated (green and purple respectively) in basic differentiation medium for, 7 days [A], 14 days [B] and 21 days [C], for being exposed to complement mediated cell lysis. PDFF cells (blue) were included as a positive control. Each graph represents a single experiment containing six replicates. Error bars represent the standard deviation of the individual observations.

5.3 Discussion

As predicted, by the well-characterised properties of germ line- and epiblast-specific telomerase expression (Wright et al., 1996), the hTERT/α1,3Gal transgene has been successfully expressed and regulated in M2 cells. The transgene provided strong expression in undifferentiated human ES cells, which was consequently down-regulated upon differentiation, mimicking expression at the endogenous hTERT locus (Wright *et al.,* 1996). The results presented here and in Chapter 4, provide clear evidence that M2 cells are susceptible to complement-mediated lysis specifically when undifferentiated, and that they lose this susceptibility with time in differentiation, as a consequence of a loss in α1,3Gal expression. In the context of regenerative medicine this could provide a powerful tool for protection against contamination of therapeutic cell types with undifferentiated human ES cells, by exposing differentiated populations to active human serum prior to engraftment.

The treatment of high-level M2 contamination with a single 1-hour exposure to active human serum, showed low level evasion of M2 cells from complement-mediated attack. This result was anticipated in a variegated population (see Chapter 3, Figure 3.2.12), where subsets of cells were not expressing the α-gal epitope at the time of incubation. However, it may be possible to overcome this issue, by using multiple exposures to active serum and complement to completely eliminate such cells prior to engraftment. Low-level contamination, on the other hand, where minority populations of M2 cells were mixed with α-gal negative HEK 293 cells were efficiently eliminated by a single 1-hour exposure to active human serum. When M2 cells constituted just 0.8% of the total population, an average of 308

colonies were observed following treatment with heat inactivated serum. By contrast the same cell populations exposed to active human serum, resulted in no ES colonies being detected after 7-10 days of ES specific culture (Figure 5.2.6 and Table 5.2.1). Similar results were also observed with mixed populations including up to 12.5% M2 contamination. From three independent experiments, only a single colony from all experiments was ever observed for populations containing, 1.6, 3.15, 6.3 and 12.5% M2 contamination, compared to an uncountable number of colonies (>500) for each replicate population exposed to heat inactivated serum (Figure 5.2.6 and Table 5.2.1). These data show that in practice M2 cells that avoid complement-mediated attack due to loss of α-gal expression, appear at low frequency, suggesting further that it was probably either the ratio of serum to target cell number or the occurrence of cell aggregates that prevented the complete lysis of M2 human ES cells, and that a second exposure to active serum would be likely to be fully effective.

The advantage of using α-gal in a clinical setting as an epitope to mediate complement attack is that humans possess high titres of naturally circulating anti-α-gal antibodies (Galili *et al.,* 1984, 1985). This could provide constant surveillance against not only cells that have evaded the initial treatment, but also against cells that dedifferentiated, or became cancerous, switching on the hTERT promoter and reactivating the α1,3Gal transgene. Furthermore, targeting the α1,3Gal transgene to acquire the endogenous hTERT promoter or targeting the construct into a characterised "neutral" site, using homologous recombination could eliminate the risk of cells evading complement-mediated lysis as a result of variegation.

A criticism of this research is that all of the data provided are based on the use of serum from blood group A individuals. Dr H. Priddle made an early attempt to lyse α-gal positive cells with serum from blood group -A and -B volunteers. In these preliminary experiments it was observed that blood group-A serum provided elevated levels of lysis as compared to blood group-B serum. This was not an unexpected result based on evidence that the anti-blood group-B antibody, contained within blood group-A serum, cross reacts with the α-gal epitope, thereby enhancing any complement-mediated response (Galili et al., 1987a). It is hypothesised that blood group-B serum would initiate a complement-mediated attack, but a longer exposure time (or multiple exposures) would be necessary. Further studies are required to evaluate the use of all blood types to initiate an α-gal-mediated complement attack, using the optimised Calcein-release assay as a method of detection.

A further criticism of this work reflects the use of a single human ES cell line, H9. Although the effects of the hTERT/α1,3Gal transgene have been studied in two independent clonal cell lines, both cell lines were derived from the H9 cell line. To support the potential of this strategy for clinical development, the transfection of other parental human ES cell lines, such as H1 and H7, would be required, to determine if the effects of complement-mediated lysis against the α-gal epitope vary.

As discussed, a critical aspect of this elimination strategy was the regulation of the α1,3Gal transgene in differentiated M2 cells. This chapter provides evidence that the α-gal epitope is down-regulated as differentiation progresses, and that loss of α-gal

epitopes at the cell surface corresponds with evasion of complement-mediated lysis. A report by Tzukerman *et al,* (2000), demonstrated that loss of hTERT promoter activity would occur after 14-days of differentiation. The data provided here shows that after 14 days of non-specific differentiation, 25% of cells continued to express α-gal epitopes from the hTERT promoter. By 21 days this population had decreased further, constituting just 8%. One hypothesis for the persistence of α-gal expressing cells following differentiation, as an alternative to residual ES cell contamination, was the presence of hTERT expressing cells within the differentiated population.

Although telomerase expression is considered a characteristic of embryonic, germ-line or malignant tissues, there is a growing body of evidence that shows expression of telomerase in some somatic cell types including, hematopoietic progenitors, particularly lymphoid lineage committed progenitors, normal peripheral blood leukocytes (Hohaus, *et al.,* 1997; Weng, *et al.,* 1996) and in the continuously proliferating epidermis basal layer (Harle-Bachor *et al.,* 1996). Non-specific differentiation protocols were employed in this investigation to determine a global picture of α-gal regulation with differentiation. However, it would be interesting to determine the level of α-gal expression following specific differentiation into osteogenic or neural lineages for example. If the persistence of hTERT expression by progenitor or mature cells was responsible for the continued α-gal expression observed with non-specific differentiation, than in populations known to lack telomerase expression, a more rapid down-regulation of α-gal would be anticipated.

Additionally, now that proof of principle has been demonstrated, a number of α1,3Gal constructs, utilising different ES specific promoters, require evaluation. One candidate promoter would be the Oct-4 promoter, which is strongly expressed in undifferentiated human ES cells and has been reported to be effectively down-regulated with differentiation (Zaehres *et al.,* 2005; Matin *et al.,* 2004; Hay *et al.,* 2004; Reubinoff *et al.,* 2000). Another candidate promoter for this comparison study would be Rex1, used by Eiges *et al.,* (2001) in a reporter construct to mark undifferentiated human ES cells. The authors demonstrated that when human ES cells were differentiated as EB's in suspension for 20 days, GFP expression, driven by the Rex1, promoter was practically eliminated. They also demonstrated that monolayers of Rex1/GFP human ES cells significantly reduced GFP expression when partially differentiated, although the time of differentiation was not specified (Eiges *et al.,* 2001).

In a recent report Martin *et al.,* (2005) have expressed caution regarding the use of human ES cells, which have been grown in current culture conditions, for human therapy. The authors demonstrated that human ES cells grown in medium containing serum replacement, acquire expression of an immunogenic, non-human sialic acid (Neu5Gc) on their cell surface (Martin *et al.,* 2005). They claim that when such cells were exposed to human serum *in vitro*, there was an increase in both C3b deposition (from 1% to 37%) and complement-mediated cell death (from 40% background to 60-70% specific) as detected by propidium iodide uptake (Martin *et al.,* 2005). However, the report indicated difficulties in determining the level of complement-mediated lysis of human ES cells, with high levels of non-specific cell death (40%)

in untreated cells, the optimised Calcein-release assay reported here shows less than 20% background. Based on the evidence in Chapter 4, the high background cell death was likely to have been caused by the dilution of human serum in GVB^{2+} buffer, see Chapter 4 (Figures 4.2.2 & 4.2.3) leading to increase human ES cell death. When complement-mediated lysis was performed on monolayer cultures, the report indicated only a 4% increase in the level of PI uptake between background and specific lysis using serum containing high natural titre Neu5Gc antibody (Martin *et al.,* 2005). This increase could be explained by intra-experimental variation due to the use of diluted serum (25% in GVB^{2+}) as indicated in Chapter 4 (Figures 4.2.6 & 4.2.14). By contrast to Martin *et al* (2005), wild type H9 ES cells used in this investigation, grown under similar conditions (in the presence of serum replacement), were not observed to be sensitive to complement-mediated lysis. Levels of Calcein-release from H9 cells exposed to active human serum were comparable to the levels observed when exposed to heat inactivated serum and a result of spontaneous release. Furthermore, when cells were exposed to a 10-fold increase in the amount of Neu5Gc reported to be present in serum replacement (0.3mM), by incubation with 3.0mM recombinant Neu5Gc, there was no increase in the observed level of calcein-release compared to untreated H9 cells (data not shown). This result suggests two possible conclusions, either that the levels of complement-mediated lysis attributed to the presence of Neu5Gc were over estimated as a result of a sub-optimal method of detection, or that the natural Neu5Gc antibody titre of the serum used in this study was insufficient to mediate complement attack. However, in the absence of commercial sources of anti-Neu5Gc antiserum a definitive conclusion could not be reached.

5.4 Conclusion

In conclusion, the data presented here and in Chapter 4, provide early exemplification of a new approach for the protection of patients against the associated risk of tumorigenicity from differentiated human ES cells, contaminated with a minority undifferentiated population. The approach could be further improved using homologous recombination, to target the transgene into a site free from position effect variegation. The production of homozygous cells, containing two copies of the transgene, would guard against gene mutation and chromosomal loss. However, it is necessary to evaluate this approach on a number of other human ES cell lines, to determine that the results are reproducible between cell lines, and to prove its worth as a widely applicable approach for the removal of contaminating undifferentiated human ES cells.

The obvious advantage of using α1,3Gal to provide unique epitopes on the cell surface of undifferentiated ES cells, is the potential for continued surveillance once engrafted. Natural immunity to α-gal in humans provides a safe-guard not only against the accidental transplantation of undifferentiated ES cells, but also against cells which dedifferentiate, or which become malignant in vivo, with reactivation of the hTERT promoter, which occurs in over 80% of studied malignancies (Tzukerman *et al.,* 2000), resulting in re-expression of α1,3Gal by these cells.

CHAPTER 6 OPTIMISING FLUORESCENCE ACTIVATED CELL SORTING OF HUMAN ES CELLS

6.1 Introduction

Fluorescence activated cell sorting (FACS) has the potential to enhance many applications in the field of stem cells research. One such area is in directed differentiation where sorting for markers of differentiated human ES cell derivatives could lead to the isolation of a minority population which could subsequently be expanded and used in therapy. Similarly, FACS could be used to deplete undifferentiated human ES cells, with tumourigenic potential, from mixed populations, as an alternative to complement-mediated lysis described in Chapters 4 and 5.

Successful application of FACS with human ES cells has been reported. Eiges *et al.,* (2001), demonstrated the identification of pure populations of undifferentiated human ES cells transfected with a GFP reporter gene, using the Rex-1 promoter to

drive expression specifically in undifferentiated and not in differentiated derivatives of human ES cells (Eiges *et al.,* 2001). The authors indicated that the use of FACS had not affected the morphology and growth of the human ES cells, but they had not assessed the pluripotentiality and karyotypic stability of the sorted population, nor had they attempted to single cell clone the sorted cells.

In this chapter the effects of FACS on undifferentiated human ES cells and their differentiated derivatives, in terms of the effects on their proliferative capacity, differentiation potential and karyotypic stability have been investigated. The rationale for doing this work was through necessity, since the BD FACSAria was a new piece of equipment, and consequently there was limited knowledge as to how human ES cells and their derivatives would respond to the process of cell sorting. Also presented within this chapter are data to support the use of FACS to successfully isolate single-cell clones of human ES like-cells, at relatively high frequency, contradicting current perception (Amit *et al.,* 2000; Thomson *et al.,* 1995).

6.1.1 Chapter Aims

1. To become familiar with and optimise the mechanics of cell sorting for human ES cells using the BD FACSAria.

2. To calibrate the FACSAria to determine the reliability and reproducibility of the data produced by comparing them with data sets acquired by flow cytometry using the BD FACScan.

3. To determine whether the process of cell sorting affects the proliferation, karyotypic stability and pluripotentiality of human ES cells.

4. To investigate the potential of FACS to isolate single human ES cell clones, using H9-ES cells carrying a GFP reporter under the control of the Oct-4 promoter.

6.2 Results

The Becton Dickinson (BD) FACSAria cell sorter was a new piece of equipment acquired during the course of this investigation and thus, before it was used experimentally the machine was assessed for stability and calibrated against data sets acquired for human ES cells using the BD FACScan cell analyser. To do this data for forward (FSC) and side (SSC) scatter and fluorescence levels from the expression of the endogenous cell surface markers, TRA-1-81 and SSEA-1, from undifferentiated human ES cells were collected on multiple occasions on both cytometers and compared.

The data presented in Table 6.2.1 and Figure 6.2.1, are representative of three independent experiments, and show that the data observed using the BD FACScan cell analyser (Figure 6.2.1A) were comparable to the data produced by the BD FACSAria cell sorter (Figure 6.2.1B). Results from both machines showed a transient increase in forward scatter (FSC), following 5 days in differentiation medium, which was lost when cells have been differentiated for 10 days. Furthermore, TRA-1-81 expression, a characteristic marker of ES and EC cells, was highly expressed in undifferentiated human ES cells, but as expected this expression was lost with increased time in differentiation (Figure 6.2.1 and Table 6.2.1.) Conversely, SSEA-1 expression, which is associated with differentiation, was up regulated after 5 days of differentiation. However, following 10 days in differentiation medium, expression appeared to drop off (Figure 6.2.1 and Table 6.2.1). This result was consistent with the transient expression reported by Draper *et al.*, 2002.

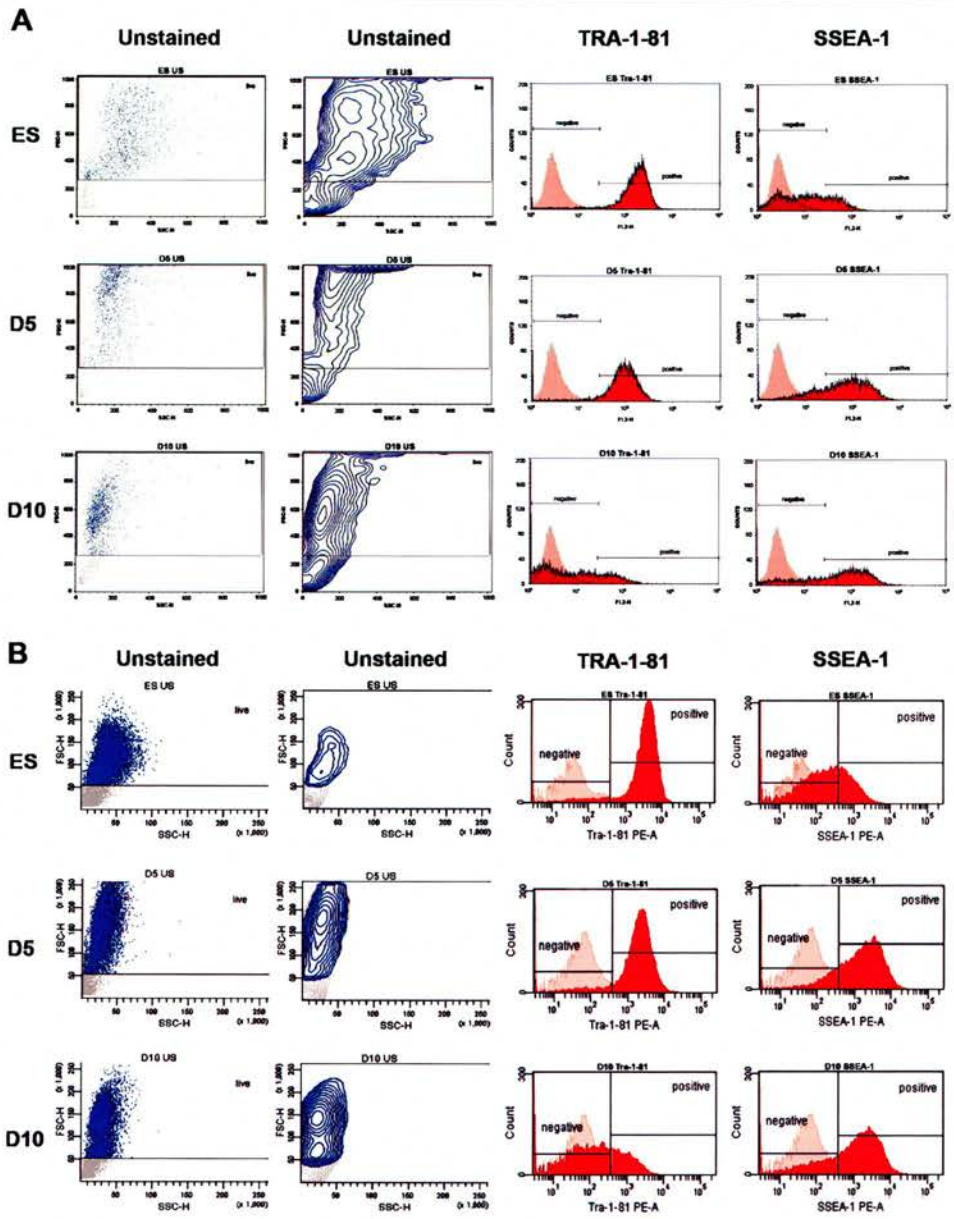


Figure 6.2.1: A comparison of the acquisition parameters for the Becton Dickinson FACScan (A) and FACSAria (B), using FSC, SSC and expression of TRA-1-81 and SSEA-1 on undifferentiated human ES cells. Images are representative of 3 independent experiments, indicating similar expression patterns from both cytometers; a transient increase in FSC after 5 days of differentiation which is lost by 10 days, progressive loss of TRA-1-81 with increased differentiation and a transient increase in SSEA-1 expression following 5 days of differentiation which appeared to be decreasing after 10 days. Contour lines represent a 70% log decrease in cell number. Faded histograms represent staining from secondary antibody controls, and all graphs represent a minium of 10,000 live events.

Positive for TRA-1-81	Undifferentiated ES	5 Days Differentiation	10 Days Differentiation
FACScan	95.4%	91.6%	22.3%
FACSAria	91.3%	88.0%	28.4%
Positive for SSEA-1	**Undifferentiated ES**	**5 Days Differentiation**	**10 Days Differentiation**
FACScan	25.2%	75.4%	67.4%
FACSAria	31.1%	80.8%	71.2%

Table 6.2.1: Data acquired for the level of cell surface marker expression on undifferentiated ES cells and ES cells differentiated for 5 and 10 days are comparable when collected on the FACSAria with data collected on the FACScan. Figures represent the proportion of positive events from a total of 10,000 live events.

It is interesting to note that the BD FACScan appears, visually, to provide clearer separation between positive and negative peaks than the BD FACSAria (Figure 6.2.1), providing greater clarity. However, when the statistics were analysed, it is clear that although the BD FACScan provides visually clearer results, statistically the two cytometers were recording similar data. A possible explanation for this is that the BD FACScan records using a logarithmic scale based on 1024 channels, as a consequence small differences appear greater. The BD FACSAria on the other hand, actually records on a linear scale based on 256,000 channels, so small differences don't appear so great, providing more accurate data. Furthermore, the BD FACSAria measures events based on the height of the electronic pulse and its duration to provide a measurement of area, the BD FACScan, only records the height of the pulse. Together with the use of a 5-log axis this makes the BD FACSAria a more sensitive machine than the BD FACScan.

6.2.1 The Mechanics of Cell Sorting does not Affect the Proliferation or Differentiation of Human ES Cells

The use of flow cytometry as an alternative method for the elimination of undifferentiated human ES cells from a mixed population first required an assessment of the ability of human ES cells and their derivatives, to survive the process of cell sorting. The rationale behind this assessment was that if undifferentiated human ES cells were efficiently removed from a mixed population, there would be confidence that the result arose because human ES cells had been specifically removed, and not because human ES cells simply had not survive the sorting process.

Differentiated and undifferentiated human ES cells were harvested as previously described (see section 2.4) using TEG solution to achieve a single cell suspension. After the initial centrifugation the cells were resuspended in culture medium and left at room temperature for 10 minutes, to allow small clumps of cells to settle out. The supernatant was then collected, pelleted and re-suspended in 200-500µl of culture medium supplemented with antibiotic (100U/ml penicillin and 100µg/ml streptomycin) to prevent growth of any opportunistic bacteria. Viable cells were then sorted based on their FSC and SSC profiles, using a gate defined as "live" to exclude dead cells and debris (Figure 6.2.2). The sorting process was designed to collect $2x10^6$ viable cells into each of two 5ml polystyrene tubes (Falcon) containing 1ml of culture medium supplemented with antibiotic. After sorting, data for a small sample (2-5,000) of cells was reacquired back through the cytometer to determine the profile of sorted cells as a way of assessing the success of a sort (Figure 6.2.2).

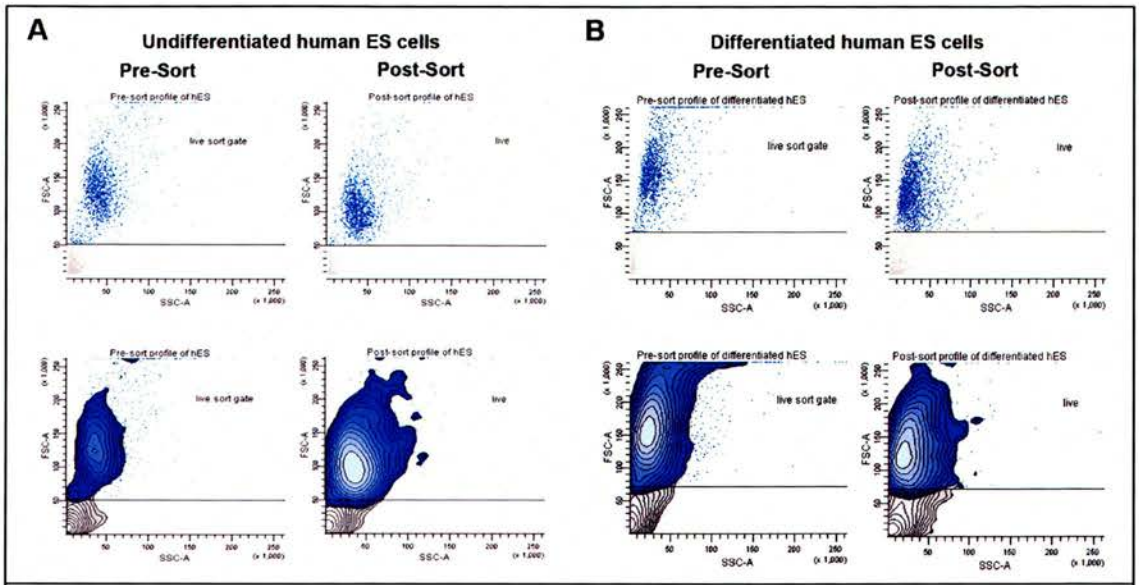


Figure 6.2.2: Undifferentiated human ES cells (A) and their differentiated derivatives (B) were sorted based on their forward (FSC) and Side (SSC) scatter, using a gate to exclude dead cells and debris - "live gate". The acquisition of post-sort data showed profiles similar to those of pre-sorted cells. In all cases the post-sort profiles display a drop in FSC and continue to show signs of cellular debris. Plots are representative of duplicate experiments for each cell state. Contour lines represent a 70% log decrease in cell number.

In all cases, the sorted undifferentiated ES cells (Figure 6.2.2A) and differentiated ES cells (Figure 6.2.2B) had a lower FSC than pre-sorted cells. The exact reason for this drop in FSC is currently unknown, although it is thought to be a consequence of changes in osmolarity, as a result of sorting cells from culture medium into a minimal volume of culture medium, which was subsequently diluted during the course of the sorting with sheath fluid, composed mainly of PBS.

From this initial data, undifferentiated human ES cells appeared to be more amenable to cell sorting than their differentiated counterparts (Figure 6.2.2). A typical pre-sort preparation of undifferentiated human ES cells contained an average

of 68% viable cells which, despite a drop in FSC, increased to 87% on average in the post-sort analysis (Table 6.2.2 and Figure 6.2.2A). The pre-sort preparations of differentiated human ES cells on the other hand contained a greater percentage of viable cells to begin with, on average 78%, although a proportion of these cells were very large and could not be represented on the dot and contour plots using the same acquisition settings. It is probably that these larger cells represented doublets or small aggregates of differentiated cells that had not been fully disaggregated, since trypsin is unable to disrupt tight junctions that form between differentiated cells. The characteristic drop in FSC as a consequence of cell sorting allowed the inclusion of these "larger" cells into the viable post-sort cell population. However, despite this, the post-sort population contained on average only 73% viable cells, suggesting that an increased level of cell death had occurred in the differentiated population compared with the undifferentiated population. It is possible that changes in osmolarity, responsible for the reduction in FSC, had a more influential effect on the differentiated ES cells as a consequence of the increased level of mechanical stress that is exerted on them as compared to undifferentiated human ES cells. The pressurised environment of the sorter may have assisted in the separation of cell aggregates but as a result could have increased the friability of the cells leading to an increased level of cellular debris (Table 6.2.2 and Figure 6.2.2B).

Cell Type	Pre-sort viability	Post sort viability
Undifferentiated human ES cells	68%	87%
Differentiated human ES cells	78%	73%

Table 6.2.2: Undifferentiated human ES cells, survive the process of cell sorting more efficiently than their differentiated derivatives. Figures represent the average proportion of cells present in the "live" gate pre- and post-sorting.

Following the sort process, the cells were pelleted to remove traces of sheath fluid and resuspended in KO-DMEM for counting. Triplicate counts, using 0.5% trypan blue, from each tube were made before the cells were plated under normal culture conditions. Cell recovery was very much lower than expected with an average of 46.72% discrepancy between the digital count provided by the BD FACSAria and the manual cell counts (Table 6.2.3).

Initially it was considered that the loss of cells might have been as a consequence of the yield and purity settings being used on the FACSAria. When set for purity, the BD FACSAria looks at the position of a cell within each drop of sheath fluid. If the cell appears close to the edge of a drop the cytometer includes the drop that precedes or follows it, depending on its position, to ensure purity. If this were the case it would mean that for every cell, two events would have to be counted by the BD FACSAria, which could have accounted for the discrepancy in cell recovery. However, when the purity setting was inactivated, cell recovery did not improve (data not shown), suggesting that the machine settings were not responsible for the poor cell recovery.

Replicate	Cell Count by FACSAria	Cell count by haemocytometer	% Cell recovery	Viability
ES Rep 1	4.5×10^6	1.71×10^6	38.00	91.50
ES Rep 2	4.5×10^6	2.47×10^6	54.89	94.60
ES Rep 3	2.0×10^6	1.41×10^6	70.50	nd
ES Rep 4	3.0×10^6	1.87×10^6	62.33	nd
Average			**56.43%**	**93.05%**
Diff Rep 1	3.0×10^6	0.94×10^6	31.33	86.00
Diff Rep 2	4.0×10^6	1.08×10^6	27.00	91.00
Diff Rep 3	5.0×10^6	2.21×10^6	44.20	nd
Diff Rep 4	2.0×10^6	0.92×10^6	46.00	nd
Average			**37.13%**	**88.50%**

Table 6.2.3: Cell recovery following sorting for cell viability (BD FACSAria) was significantly lower than expected. Expected values were based on the BD FACSAria's internal digital counter. Following sorting, a manual cell count using 0.5% trypan blue staining and a standard haemocytometer revealed a value significantly lower than expected level of cell recovery. Triplicate counts were made for each sample, the top portion of the table refers to undifferentiated human ES cells, while the bottom refers to differentiated human ES cells, indicating a significant difference (p=0.02) between the two cell states. nd denotes no-data.

It is possible that poor cell recovery was a consequence of cell shearing when the cells hit the surface of the medium within the collection tube, or through the adhesion of cells to the polystyrene collection tubes, used in these experiments. Alternatively, the pressure exerted by the BD FACSAria, could have had a detrimental effect on cell survival and could reflect inherent difference between different cell types. Undifferentiated human ES cells had a significantly higher (p=0.02) level of cell recovery than their differentiated counter parts (Table 6.2.3) suggesting that perhaps the pressure exerted on the cell aggregates or larger differentiated cells was more detrimental. It would be interesting to repeat these

experiments using medium- or low-pressure settings and polypropylene collection tubes in an attempt to improve the level of cell recovery.

Interestingly, the viability of those cells recovered, assessed by trypan blue staining, was observed to be very high, on average >90% cell viability (Table 6.2.3), suggesting further that it was an aspect of the sorting process that was resulting in cell loss.

6.2.1.1 Plating efficiency and morphology are comparable

Following cell sorting, both differentiated and undifferentiated human ES cells were re-plated at $4.5x10^5$ and $8.5x10^5$ respectively in duplicate $25cm^3$ flasks. Twenty-four hours after plating, cells were recovered from one flask for counting to assess plating efficiency (Table 6.2.4). The difference in plating density between the two cell populations was a consequence of recovered cell numbers, recovery of far fewer differentiated cells than undifferentiated cells meant that it was not possible to plate the cells at the same density. The assumption was that the differentiated cells would be able to grow at this low density while current opinion suggests that undifferentiated human ES cells plated at low density would have spontaneously differentiated (Thomson *et al.,* 1998; Thomson *et al.,* 1995).

The cell counts presented in Table 6.2.4 and Figure 6.2.3, indicated little difference in the plating efficiency of sorted and unsorted cells, for both differentiated and undifferentiated human ES cells. However, there was a significant difference

between the percentage plating efficiency of undifferentiated and differentiated human ES cells, whether sorted or not. Initially it was thought that the seeding density of differentiated ES cells was too low, at $4.5x10^5$ per $25cm^3$ flask, however, when undifferentiated human ES cells were plated at densities as low as $3x10^5$, the plating efficiency remained significantly higher than that of differentiated cells (over 83% compared to 25-30% for differentiated cells).

	Undifferentiated human ES cells		Differentiated human ES cells	
	Unsorted	**Sorted**	**Unsorted**	**Sorted**
Average cells plated at p1	$8.50 x10^5$	$8.50 x10^5$	$4.50 x10^5$	$4.50 x10^5$
Average cell count (p1)	$7.18 x10^5$	$6.90 x10^5$	$1.33 x10^5$	$1.08 x10^5$
Average plating efficiency	**84.40%**	**81.25%**	**29.35%**	**23.90%**
Average cells plated at p2	$5.00 x10^5$	$3.00 x10^5$	nd	nd
Average cell count (p2)	$4.74 x10^5$	$2.58 x10^5$	nd	nd
Average plating efficiency	**94.70%**	**83.00%**	nd	nd
Average	**89.55%**	**82.12%**	**29.35%**	**23.9%**
S.D.	**11.08%**	**4.95%**	**3.25%**	**1.1%**

Table 6.2.4: Plating efficiency for both differentiated and undifferentiated human ES cells were not significantly affected by cell sorting. No data (nd) can be provided for differentiated cells at passage 2 (p2), as the cells never reached confluence they were discarded before passage. Values represent the mean of triplicate cell counts for each duplicate. Data coloured grey indicates a loss of a substantial amount of the cell pellet during passaging of one of the replicates, hence the significant reduction in cell number plated (in this instance cells were plated in a well of a 6-well plate not in a $25cm^2$ flask).

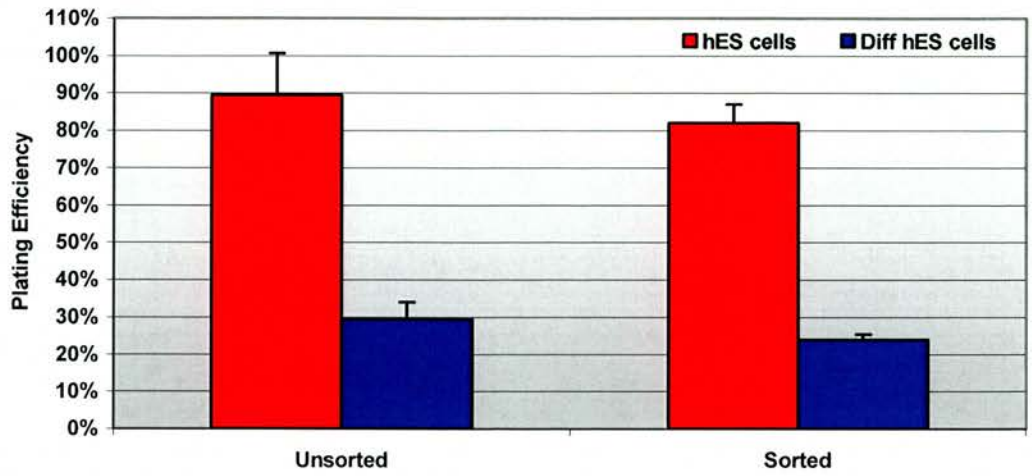


Figure 6.2.3: Plating efficiency of undifferentiated (red) and differentiated (blue) human ES cells following passage (sorted) through the FACSAria cell sorter. Cells from the same preparation were reseeded at the same density following the same period of time in suspension but without passing through the sorter (unsorted). Differentiation of human ES cells was as a monolayer in the absence of CM and bFGF for at least 14 days. Data represents the mean of triplicate cell counts from two independent experiments at two passages; error bars represent one standard deviation of the mean.

Interestingly, cultures of sorted and unsorted differentiated human ES cells contained a large proportion of dead cells. Furthermore, those cells that survived didn't proliferate extensively in the days after plating and following 8 days in continuous culture the cells had changed in morphology and were senescent (Figure 6.2.4A). Consequently they were discarded and it was decided that in future experiments the plating density for differentiated cells would need to be increased, or the surface area onto which they were plated would be decreased.

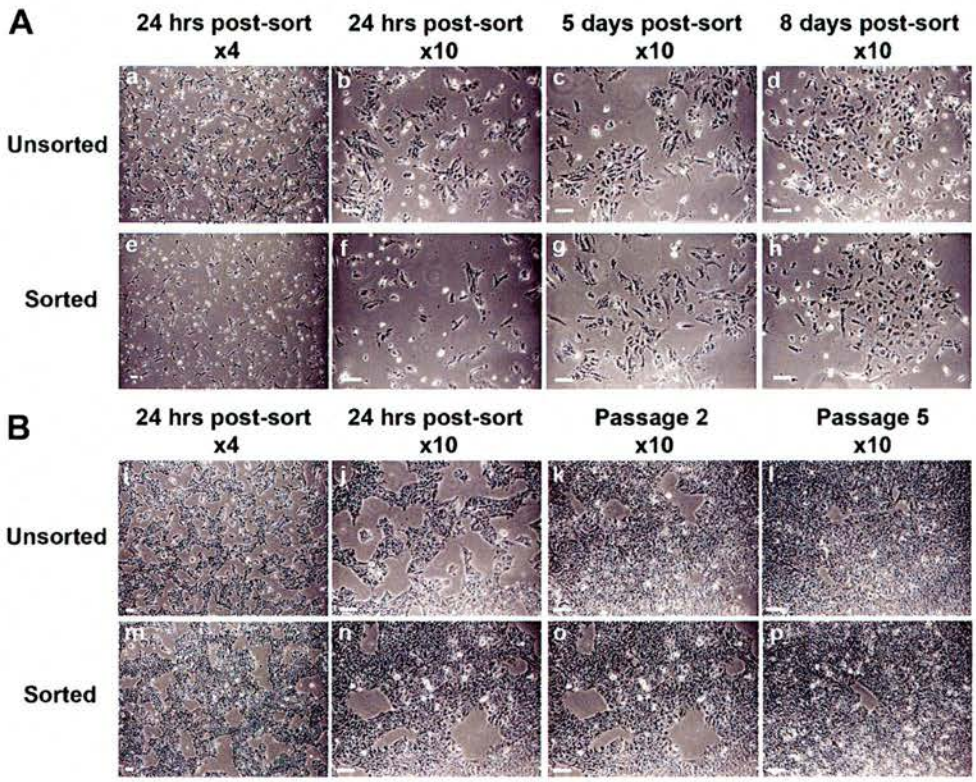


Figure 6.2.4: Differentiated (A) and undifferentiated (B) human ES cells sorted using a live gate, behave in a similar manner to their unsorted counterparts after re-plating. Images a, b, e, f, i, j, m and n represent 24 hours after plating (a, e, i and m at x4 and b, f, j and n at x10 magnification). Images c and g follow 5 and d and h follow 8 days in differentiation culture show continued differentiation, but loss of proliferation. Images k and o, and l and p, represent continued proliferation of undifferentiated human ES following 2 and 5 passages respectively. Scale bars represent 100µm.

By comparison, in excess of 89% of sorted undifferentiated human ES cells were adherent (Table 6.2.4), containing only small numbers of dead cells. Furthermore, the proliferative capacity of the cells was not impeded, with confluence being reached two-three days after plating (Figure 6.2.4B). Sorted and unsorted human ES cells were maintained in culture for a further five passages in their undifferentiated state, before being assessed for continued normality and pluripotentiality.

6.2.1.2 Karyotypic normality is maintained following the sorting process

Mitotic spreads where made from both unsorted (A) and sorted (B) human ES cells following 8 passages in culture (p99+8). A total of 30 spreads were counted from each population, 10 of which were fully analysed (Fletcher, J., Roslin Institute, Edinburgh), showing a normal 46XX karyotype (Figure 6.2.5)

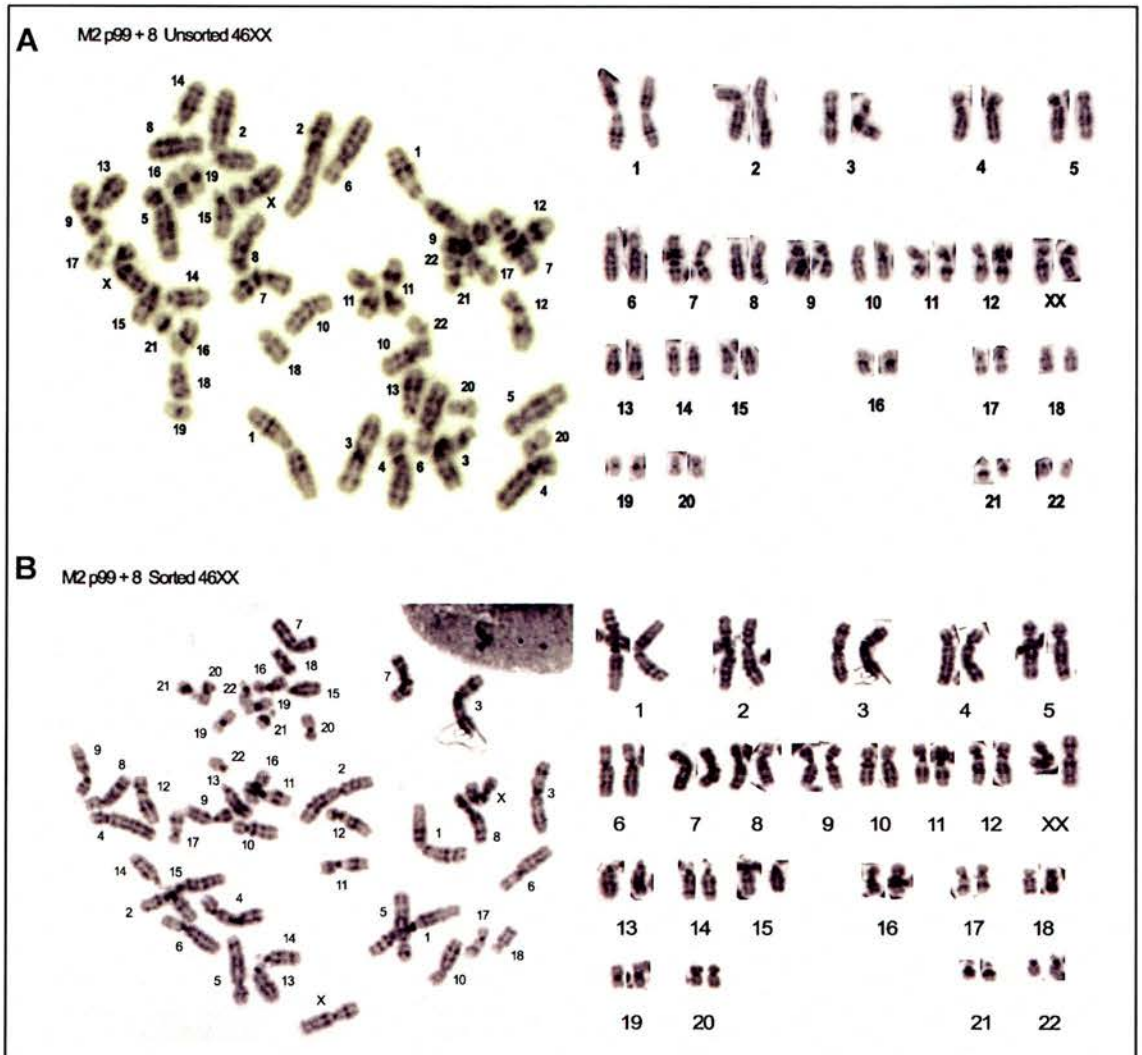


Figure 6.2.5: Representative mitotic spreads from unsorted (A) and Sorted (B) M2 cells, 8 passages after they were sorted (or mock sorted). Both cell populations have a normal 46 XX karyotype with no obvious chromosomal abnormalities. Images produced in collaboration with J. Fletcher, Roslin Institute.

6.2.1.3 Expression of ES specific markers are maintained on sorted undifferentiated human ES cells

Human ES cells have been characterised extensively in the literature by the expression of a number of markers; positive expression of the cell surface epitopes SSEA-4, TRA-1-60 and TRA-1-81, expression of Oct-4 and alkaline phosphatase and the lack of SSEA-1 expression (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998). Consequently, to assess the normality of the human ES cells, following cell sorting, both sorted and unsorted human ES cells were assessed for expression of the above panel of characteristic markers.

Expression of cell surface markers was assessed by flow cytometry (BD FACScan) and showed positive staining for SSEA-4, TRA-1-60 and TRA-1-81 to the same extent in both sorted and unsorted populations (Figure 6.2.6a, c and d respectively). As expected, both populations lacked expression of SSEA-1 (Figure 6.2.6b), indicating that sorted undifferentiated human ES cells maintained their undifferentiated state and that there had been no adverse effects on cell surface marker expression as a consequence of cell sorting.

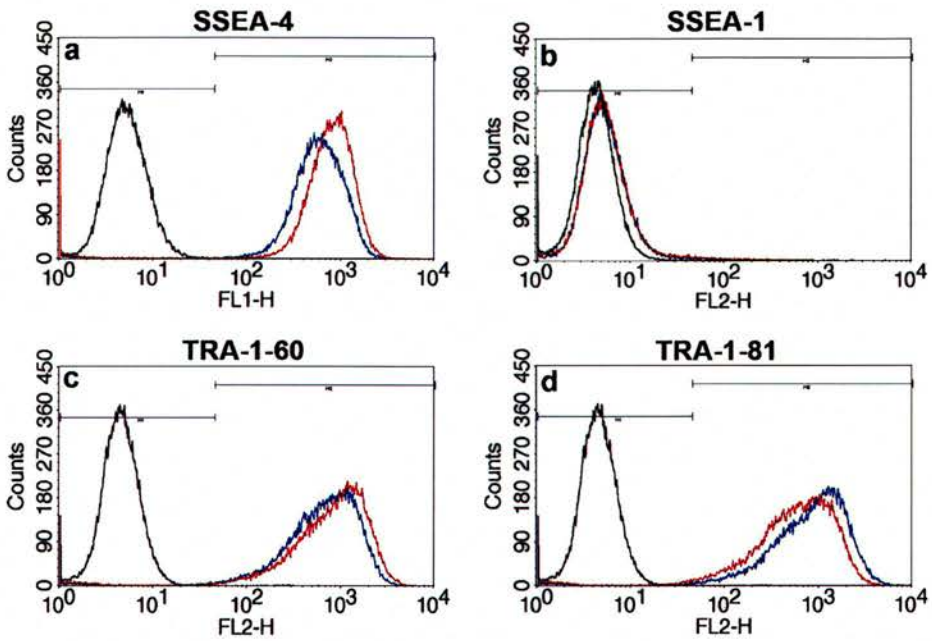


Figure 6.2.6: Cell surface expression of the markers SSEA-4 (a), SSEA-1 (b) TRA-1-60 (c) and TRA-1-81 (d) remained constant on cells subjected to the process of sorting (blue) as compared to the same cells which had not been sorted (red), after 5 passages in culture. Each data set consists of 10,000 events. The black line represents the level of non-specific binding of the secondary antibodies. FL1 – 530/30nm bandwidth filter detects FITC-stained cells, while FL2 – 585/42nm bandwidth filter detects R-PE-stained cells.

Immunochemistry for the nuclear expression of the transcription factor Oct-4 showed clear and strong co-localisation with the nuclear stain dapi in both sorted (a) and unsorted (b) human ES cells (Figure 6.2.7). Furthermore, all human ES cells expressed alkaline phosphatase as determined using the BCIP/NBT substrate IV kit (Vector Labs.) to the same extent, whether sorted (a and b) or not (c and d; Figure 6.2.8)

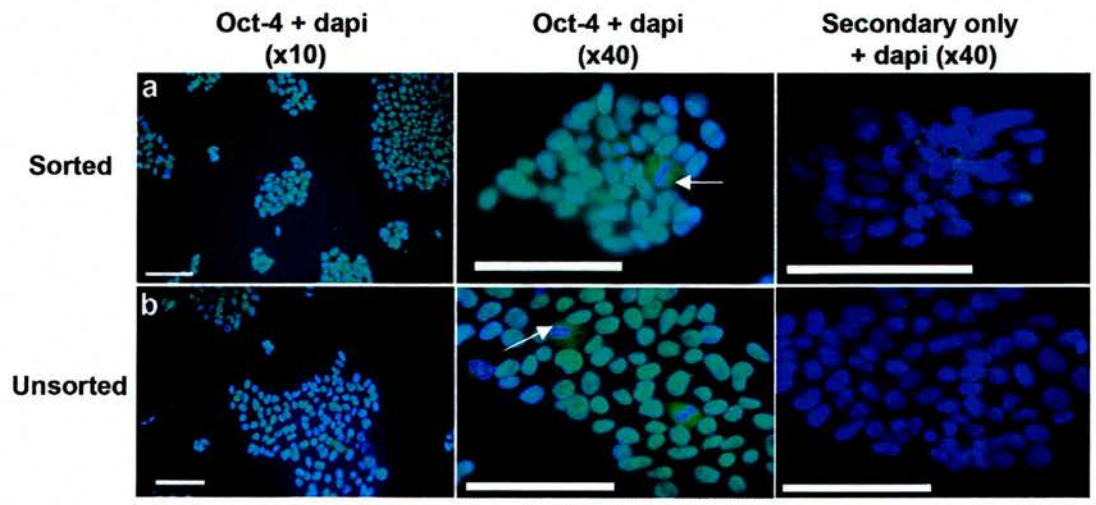


Figure 6.2.7: Cells exposed to the process of cell sorting (a) expressed Oct-4 at a level comparable to cells not exposed to the sorting process (b). Oct-4 (FITC, green) co-localises with the nuclear stain dapi (blue) to give a turquoise positive stain. In dividing cells (indicated with arrows) Oct-4 leaks from the nucleus into the cytoplasm producing a diffuse green stain. Columns 1 and 2 show positive staining at x10 and x40 magnification respectively. Column 3 shows the negative control, FITC-conjugated secondary antibody with dapi. Scale bars represent 100μm.

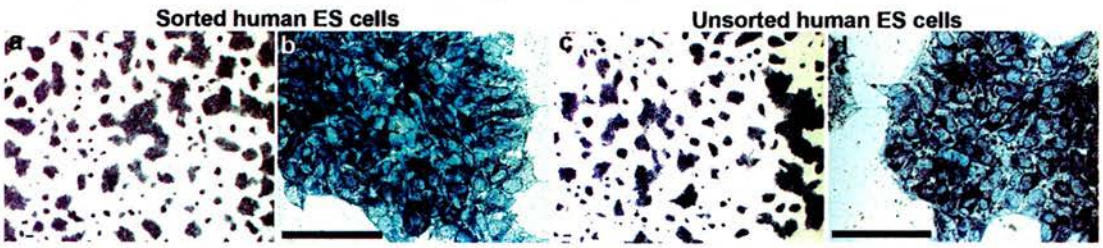


Figure 6.2.8: Positive alkaline phosphatase staining was observed in all cells whether exposed (a and b) or not (c and d) to the process of cell sorting. Images are by differential interference contrast (DIC) microscopy at x4 (a and c) and x40 (b and d) magnification. Scale bars represent 100μm.

6.2.1.4 Human ES cells, which have undergone the sorting process, can differentiate into cell types representative of the three-germ layers and can be directed to differentiate down the osteogenic pathway

In collaboration with Davina Wojtacha (Roslin Institute, Edinburgh), sorted human ES cells were assessed for their continued pluripotentiality. Using the standard undirected differentiation protocol, described in Chapter 3 (ref methods), sorted and unsorted human ES cells were formed into embryoid bodies in suspension before being plated on gelatin coated bucket slides (NUNC), and allowed to spontaneously differentiate for a further 14 days (21 days in total). The resulting cells were then analysed for markers representative of all 3 embryonic germ layers, endoderm (α-fetoprotein; AFP), ectoderm (β-tubulin III) and mesoderm (muscle specific-actin).

Figure 6.2.9 demonstrates that sorted undifferentiated human ES cells, maintained the potential to differentiate into cell types representing all 3 embryonic germ layers, with an abundance of β-tubulin III (c) and muscle specific actin (d) staining and clear areas of AFP staining (a and b). In the unsorted population, however, while there was an abundance of both β-tubulin III (f) and muscle specific actin (g), there was far less evidence of AFP staining (e) than was observed for the sorted population. Furthermore, due to the reduced frequency of AFP positive cells and because of the nature of AFP positive cells, often differentiating as 3-dimenstional structures in very dense areas of cells, it was not possible to obtain clear images showing high magnification images of AFP positive cells in the unsorted population. It is likely that the difference in AFP staining between the sorted and unsorted populations occurred by chance, and that with further repeats, using this undirected

differentiation protocol, examples would be found where there was an increased amount of AFP staining and where clearer images of AFP positive cells could be obtained. However, it is also possible that by exerting the selection pressure of sorting on undifferentiated human ES cells that a juvenile cell, with increased potentiality had been isolated.

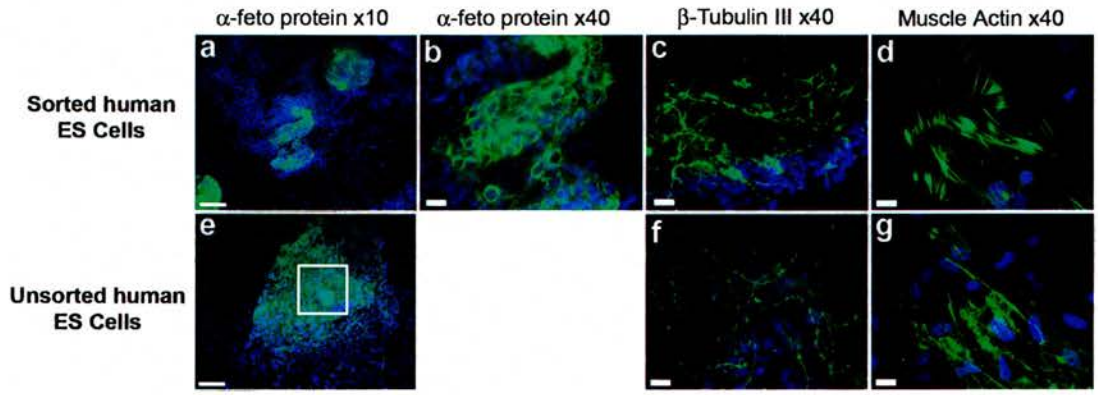


Figure 6.2.9: Sorted human ES cells maintain their potential to differentiate into cell types representative of all 3 germ layers. Sorted human ES cells (a-d) differentiated into cells that express α-fetoprotein (a x10, b x40), β-Tubulin III (c, x40), and muscle specific actin (d x40). Unsorted human ES cells readily differentiated into identifiable cell types expressing β-Tubulin III (f, x40), and muscle specific actin (g x40). However, do to the nature of AFP positive cells, they differentiate in areas of dense cells, and it was not possible to obtain images in clear focus with out background staining for the unsorted population. The area in image e, outlined by a white box, is an area typical of AFP staining, showing the characteristic sphere (x10). Scale bars represent 100μm.

In addition, the sorted and unsorted human ES cells were also directed to differentiate into bone, using the directed osteogenic protocol developed within the group (Sottile et al., 2003). Briefly, human ES cells were harvested and aggregated into EBs, as previously described, and were then plated on to gelatin, in basic

differentiation medium supplemented with the osteogenic factors β-glycerophosphate (10mM), ascorbic acid (50μM) and dexamethasone (0.1μM) (see section 2.8.5). The progress of osteogenic differentiation was assessed at 0, 3, 6, 9, 13, 16 and 20 days post induction, by Alizarin Red S staining (Figure 6.2.10 and Figure 6.2.11) and calcium deposition (Figure 6.2.12).

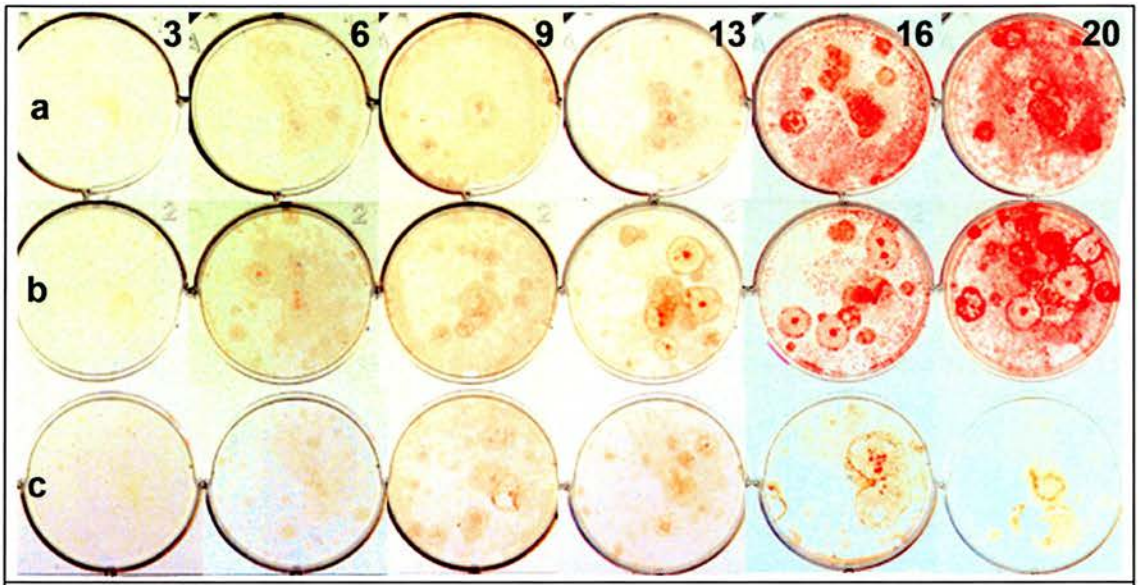


Figure 6.2.10: Sorted (a) human ES cells have the same capacity as unsorted (b) human ES cells to differentiate into bone, in the presence of osteogenic factors (a & b). Significant numbers of bone nodules started to form after 13 days in the presence of osteogenic factors, but not in the absence of osteogenic factors (c), at similar frequencies between the two populations. Images produced in collaboration with Davina Wojtacha (Roslin Institute, Edinburgh).

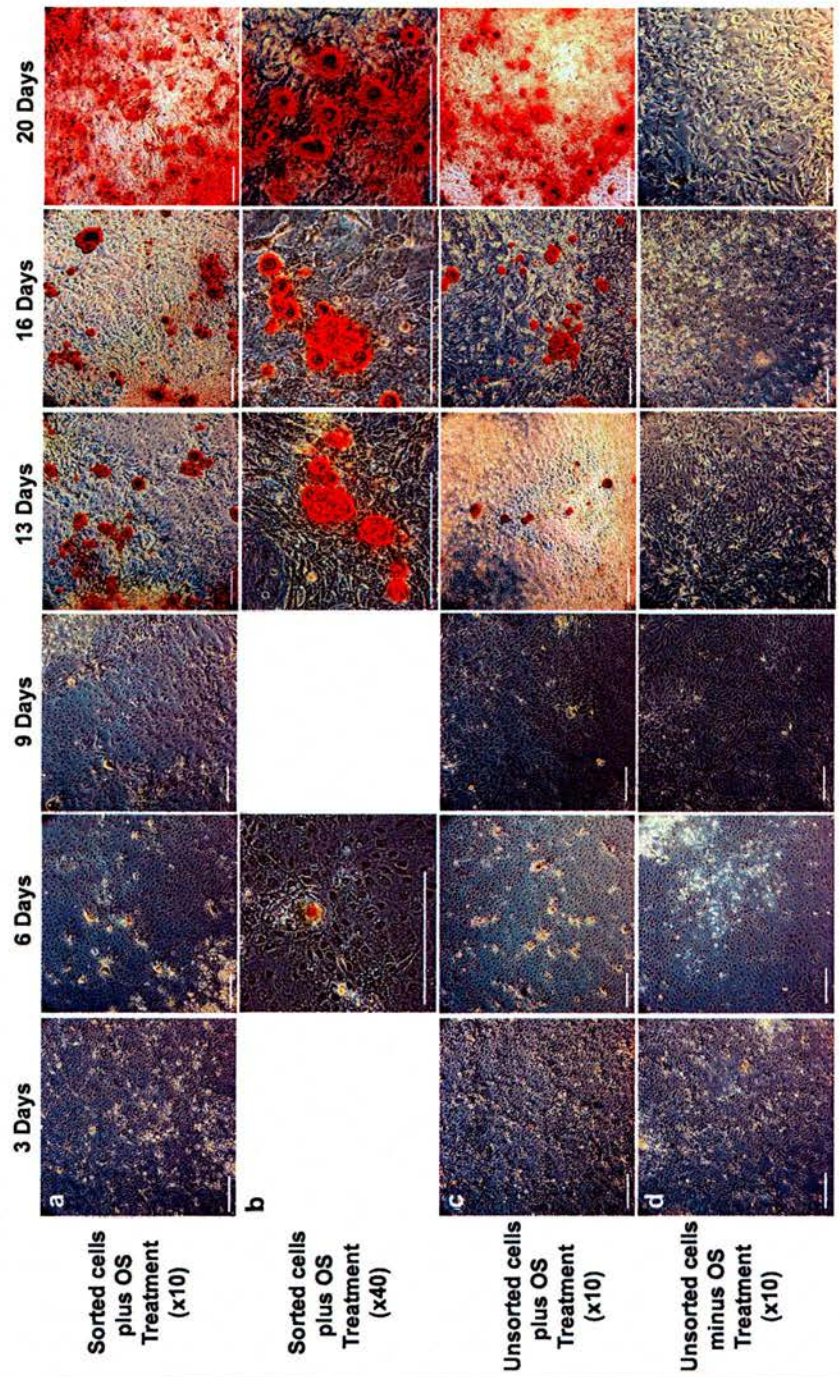


Figure 6.2.11: Phase contrast microscopy showing the development of bone nodules with time in differentiation, specifically in response to osteogenic factors (a, b and c), panel d represents no osteogenic factors. No significant difference was observed between the ability of sorted (a x10 and b x40) and unsorted (c x10) cells to differentiate into bone. Images produced in collaboration with Davina Wojtacha (Roslin Institute, Edinburgh), scale bars represent 250µm.

Alizarin Red S staining indicated an abundance of bone nodules following 16 days in osteogenic differentiation medium in both the sorted and unsorted populations (Figure 6.2.10). However, using phase contrast microscopy (x40), nodules were observed as early as day 6, in the sorted population (Figure 6.2.11b). Calcium deposition data indicated a significant increase in calcium following 3-6 days in osteogenic differentiation medium, which precedes the development of bone nodules in both the sorted and unsorted cells (Figure 6.2.12).

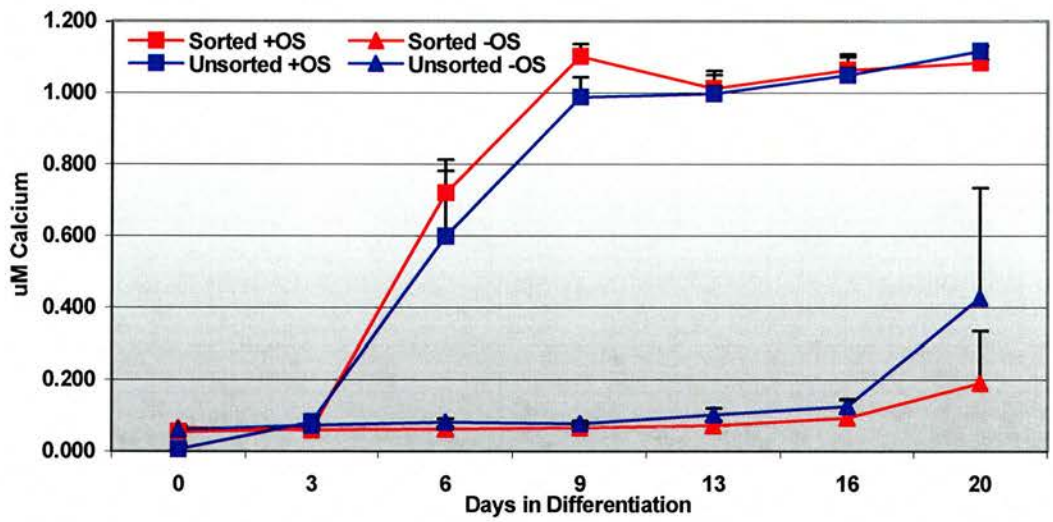


Figure 6.2.12: Calcium deposition as a result of directed osteogenic differentiation is unaffected by the process of cell sorting. Sorted human ES cells (red) deposit calcium at levels comparable with unsorted (blue) human ES cells, when differentiated in the presence (■) of ostegenic factors for 20 days. Differentiation in the absence (▲) of osteogenic factors resulted in minimal calcium deposition in both sorted and unsorted cells. Data represents the average of triplicate wells and the error bars represent one standard deviation about that mean. Data produced in collaboration with D. Wojtacha, (Roslin Institute, Edinburgh).

The behaviour of both sorted and unsorted human ES cells following osteogenic differentiation suggested that the process of cell sorting had not affected their ability to be directed to differentiate into cell types of the mesoderm germ layer. Following the results of the undirected differentiation, it would be interesting to determine if there were any significant differences in the ability of sorted and unsorted human ES cells to be directed to differentiate into cell types of the endoderm lineage.

6.2.2 FACS is an Efficient Method for Single Cell Cloning of Undifferentiated Human ES Cells.

With the evidence presented here, and the indication in Chapter 5 that, possibly, single cells can survive complement-mediated lysis, leading to the formation of a colony, it was hypothesised that perhaps cell sorting could be used to produce single cell clones of undifferentiated human ES cells. To test this hypothesis, H9 cell lines containing a GFP-reporter gene, driven from the full 8.5kb murine Oct-4 promoter were generated (see appendix I.3), and single cells sorted into 96-welled plates.

From in excess of 50 colonies, produced by lipofection (see section 2.7.1), 9 colonies were suitably discrete to enable them to be picked as a single colony for expansion into lines, of which only 6 survived expansion. When analysed by fluorescence microscopy, all 6 cell-lines, named Oct-4/GFP-3 to -7 and -9, had variegated GFP expression. Maintaining cells in the presence of 200μg G418 reduced the level of variegation (data not shown), however, there remained significant differences between the lines (Figure 6.2.13). Oct-4/GFP-6 showed the

lowest level of variegation, by fluorescence microscopy, and consequently was used in all subsequent experiments.

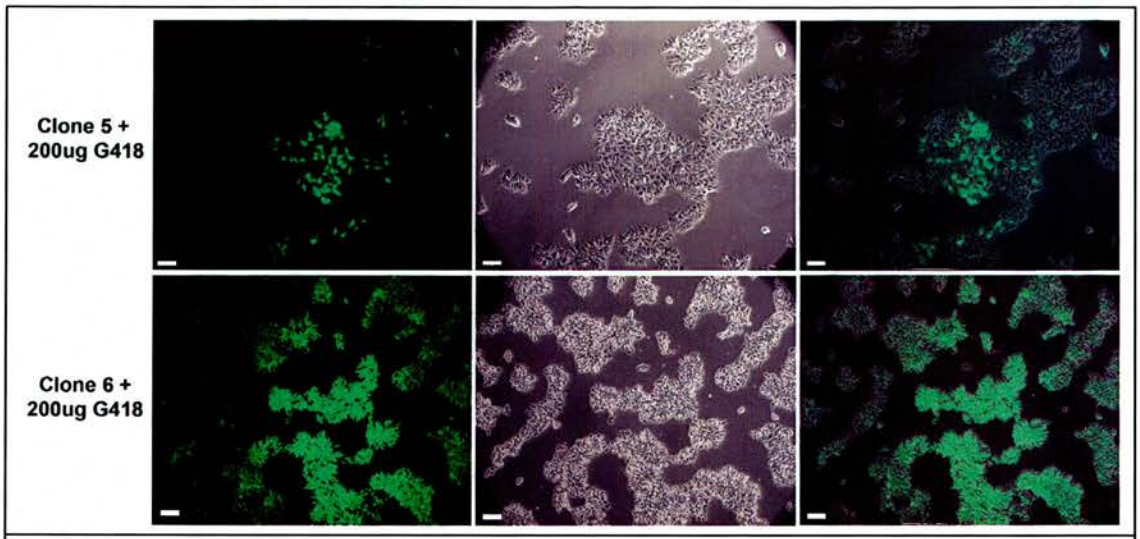


Figure 6.2.13: Oct/GFP-6 human ES cell line had a more uniform GFP expression pattern from the Oct-4 promoter, than Oct/GFP-5. Random integration of transgenes results in positional effects. Clone 5 is an illustrative example, where despite being cultured in the presence of G418 selection, expression of GFP is minimal. Oct/GFP-6 on the other hand has almost ubiquitous expression in the presence of G418 selection. Scale bars represent 100μm.

Oct-4/GFP-6 cells were first assessed for their ability to down-regulate the Oct-4 promoter when differentiated. Differentiation was induced, as previously described (see section 2.8.4), by forming embryoid bodies in suspension and seeding EBs onto gelatin coated plates in basic differentiation medium (KO-DMEM supplemented with 10% FBS (v/v), 0.1mM NEAA, 2mM L-Glutamine and 0.1mM β-mercaptoethanol). Figure 6.2.14 shows representative images of a differentiation time-course, where GFP expression was down-regulated in the majority of cells following 6 days of differentiation (Figure 6.2.14 d-f). Those cells that maintained GFP expression were located in dense areas of cells and in areas with a 3-

dimensional structure, which would often beat. This may represent continued expression, or perhaps persistence of the GFP protein (d, e, g and h) since it was the unmodified EGFP that was used with a reported half-life of 26 hours (Ward & Stern, 2002; Corish & Tyler-Smith, 1999). Following 14 days plated in differentiation medium, no convincing GFP expression was observed, only auto-fluorescence from dense areas of cells and beating cells (Figure 6.2.14j-l).

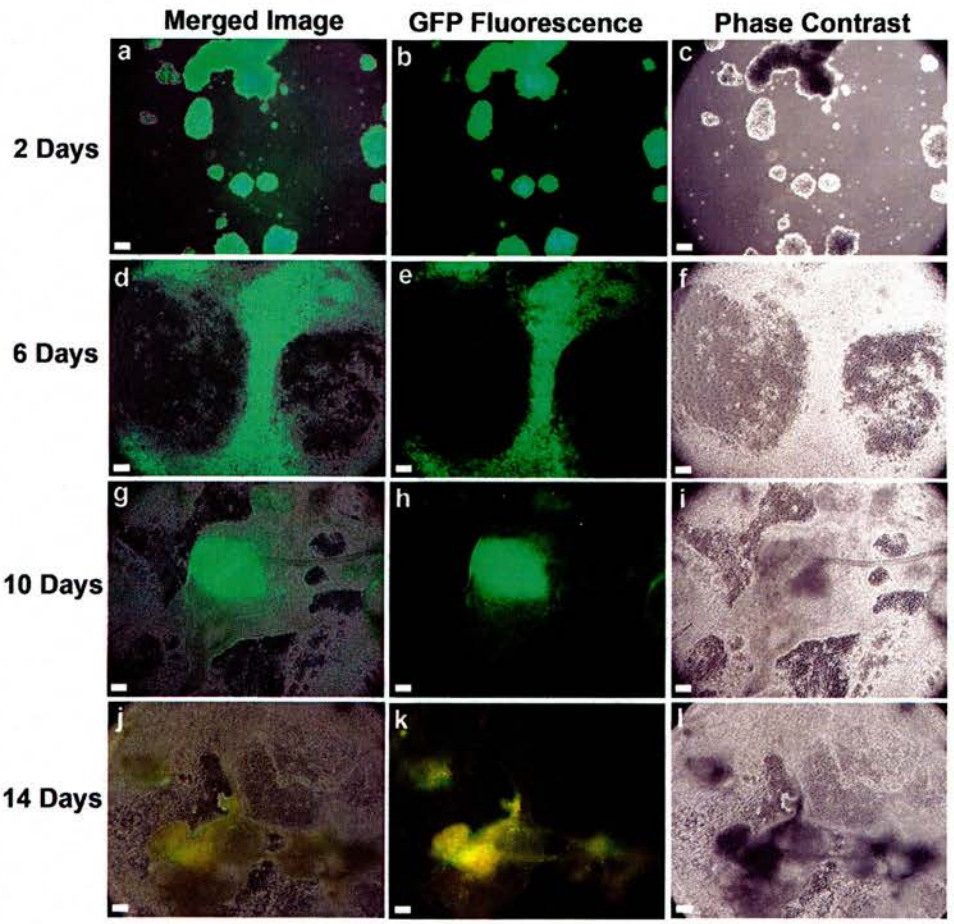


Figure 6.2.14: Oct/GFP-6 effectively down-regulated GFP expression with time in differentiation medium. The majority of Oct/GFP cells have lost GFP expression following 6 days in differentiation medium. Those cells that persist are located in dense areas and could represent either continued expression or persistence of trapped GFP protein. Following 14 days in differentiation medium the only staining observed was auto-fluorescence from very

dense areas of cells. Left panel shows merged images of the centre, GFP fluorescence and right, phase contrast images. Scale bars represent 100µm.

Oct-4/GFP-6 cells were cultured and harvested as previously described (see section 2.4), and were sorted into 4 replicate, 96-well plates containing conditioned medium supplemented with 100U penicillin and 100µg streptomycin, by Martin Waterfall (Roslin Institute, Edinburgh) using the BD FACSAria. The sort plan used for each of the 4 replicate 96-well plates, placed the wells containing the highest numbers of cells (50, 20 and 10 cells) around the edges of the plates (Figure 6.2.15 e). This was a deliberate action, as it is known that the outer wells of a 96-well plate can be affected by evaporation resulting in the loss of cells contained within.

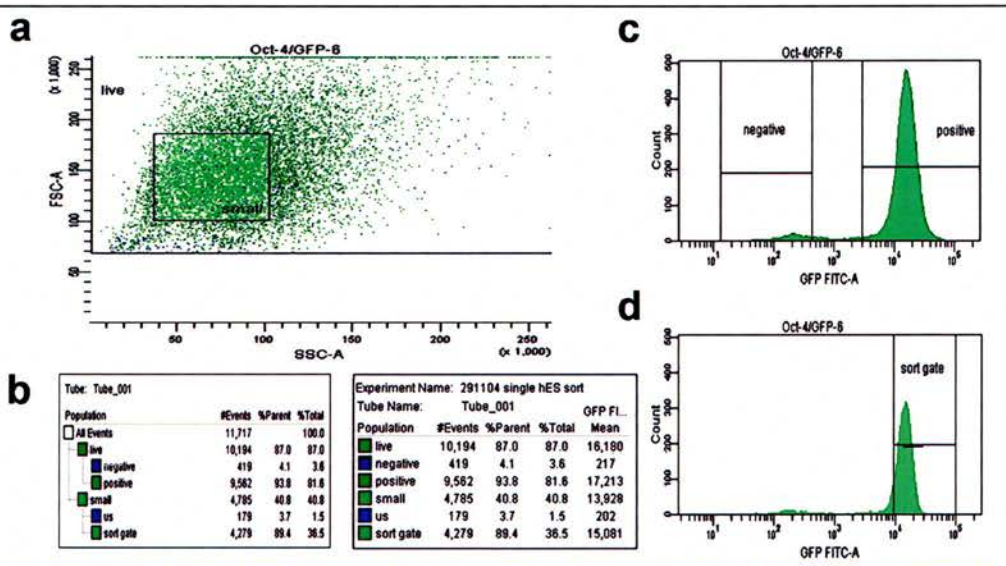


Figure 6.2.15: Human ES cells expressing Oct-4/GFP were single cell sorted into 96-welled plates based on position of FSC and SSC (live gate to remove dead cells and debris) and on positive GFP fluorescence. Dot plot (a) defines the "live" gate based on FSC and SSC and the proportions and statistics are provided (b). GFP positive and negative populations are

identified (c) to establish the range for the sort gates (d). The sort plan for the distribution of cells within a 96-well plated is provided (e).

The plates were maintained without media change for 1-week, at 37°C in a humidified incubator plus 5% CO_2, and a decision was made to make an assessment of the number of wells containing surviving cells before the medium was changed. There were 2 reasons for this decision: a) to prevent disturbance of the colonies, such that each colony was generated from a single cell deposited by sorting and not from the production of satellite cells, which would have resulted in an apparent increase in colony number, and b) to prevent any possible well-to-well contamination in the media changing process from impacting on the efficiency of cell survival. After a 1-week period in culture, a significant number of colonies were visible by phase contrast microscopy (Table 6.2.5).

No of cells per well	No of wells seed	No of wells with cell growth	Frequency of cell survival	No with GFP expression	Frequency of GFP expression in surviving cells
50	8	1	12.5%	0	0.00%
20	44	8	18.2%	1	12.5%
10	44	12	27.3%	5	41.7%
8	48	19	39.6%	12	63.2%
6	48	13	27.1%	10	76.9%
4	48	14	29.2%	11	78.6%
2	48	9	18.8%	9	100.0%
1	96	10	10.4%	9	90.0%

Table 6.2.5: Cell survival rates of single human ES cell clones are relatively high, and show strong expression of GFP driven by the Oct-4 promoter. Survival of human ES cells in the outer rows of the 96-wells were low as anticipated, and those cells which did proliferate, appeared in most cases to switch off the Oct-4 GFP construct. Data represent the absolute number of wells containing proliferating cells from 4 replicate plates.

The cell survival data in Table 6.2.5 supported the expectation that wells on the edge of a 96-welled plate were less amenable to cell growth than those in the centre. From a total of 96 wells, only 21 wells (22%) containing 10 or more cells, supported proliferating cells after a 1-week period, out of which only 6 wells (6.25%) contained cells that continued to express the Oct-4/GFP transgene following 2 weeks in culture.

Interestingly, the frequency of wells containing proliferating cells from a single seeded cell was very encouraging, with a 10.4% success rate. This frequency was a significant improvement on that reported by Amit *et al.,* (2000), who described clonally derived human ES cell lines at a frequency of just 0.52%. Furthermore, 90% of the wells that contained proliferating cells from a single seeded cell also continued to express the Oct-4/GFP transgene (Table 6.2.5 and Figure 6.2.16). However, there was some degree of variation in the morphology of the proliferating cells. Out of the 9 wells, 2 contained cells with a non-ES like morphology, suggesting that they were differentiating and perhaps in the process of down regulating Oct-4 expression (Figure 6.2.16b). However, this was not a result that was unique to the single cell wells, there was also evidence in the 2-cell (c) 4-cell (d), 6-cell (data not shown) and 10-cell (f) wells (Figure 6.2.16).

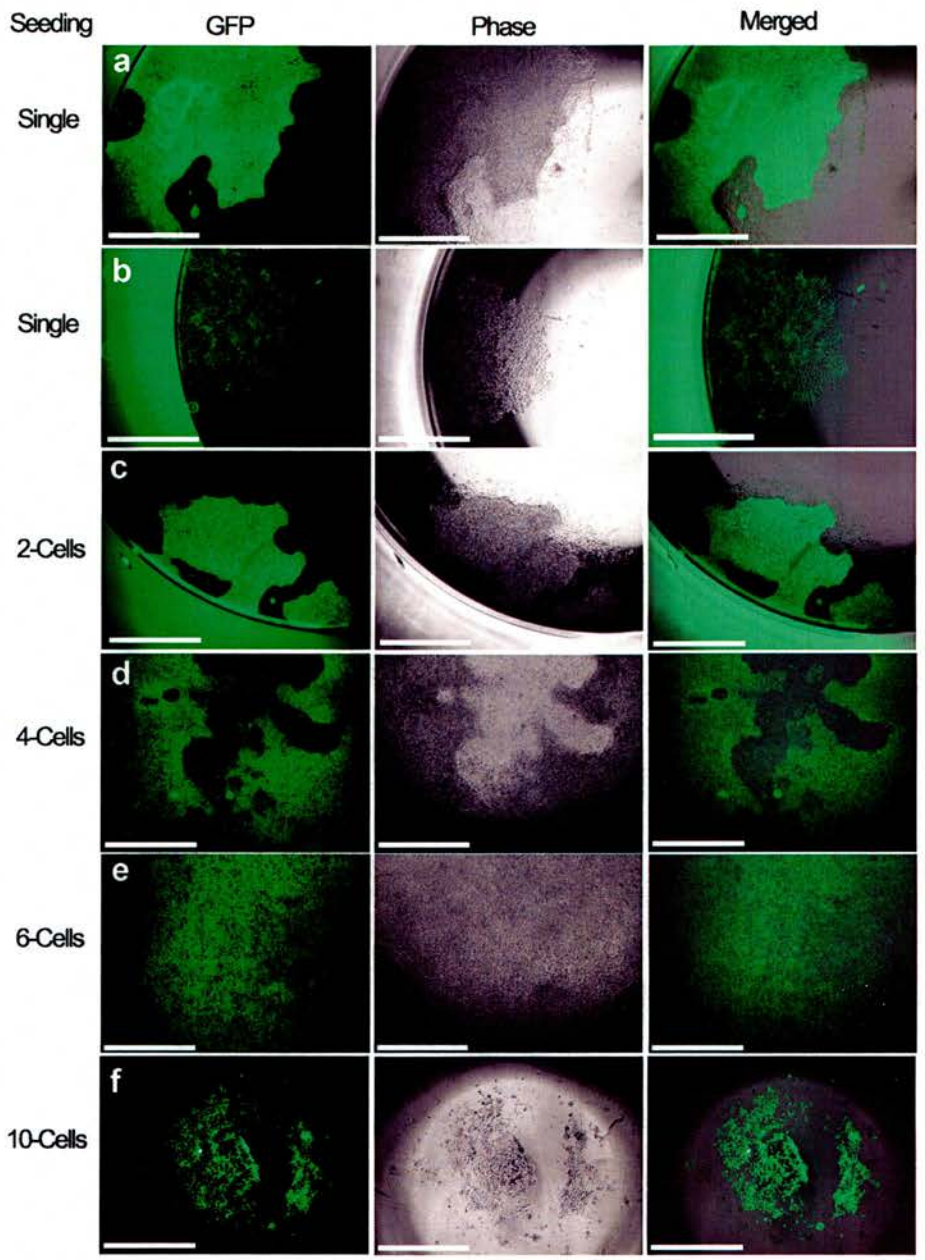


Figure 6.2.16: Representative examples of human ES cells, which have proliferated for 2 weeks after cell sorting and show expression of the Oct-4/GFP transgene. Cell which had been individually seed (a and b), 2-cells seeded (c), 4-cells seeded (d) 6-cells seeded (e) and 10-cells seeded (f) per well proliferated into visible colonies 1 week after being sorted and continued to express the Oct-4/GFP construct. The majority of surviving colonies also maintained an ES-like morphology, but some colonies, particularly those in wells around the edge of the plates, (b and f) had lost their ES-like morphology, but continued to express the Oct-4/GFP transgene. Scale bars represent 100μm

Preliminary data for the isolation of single cell human ES cell clones using cell sorting indicated that it was relatively efficient (10.4%), however, these results represent a single experiment, which must be repeated in order to confirm the observations. Furthermore, it is essential that clonal human ES cells are expanded into cell lines and characterised for karyotypic stability and continued pluripotentiality. Unfortunately, due to bacterial contamination, this was not possible at the time of this first experiment, but it will be an essential result if cell sorting is to be used to generate single cell clones of human ES cells in the future and is currently being repeated within the group.

6.3 Discussion

The data presented in this chapter indicated that the BD FACSAria could be used to reproducibly sort undifferentiated human ES cells. Furthermore, when those cells were placed back into culture, they proliferated to the same extent as those which had not been sorted and upon further characterisation, sorted undifferentiated human ES cells expressed markers, characteristic of undifferentiated human ES cells, to the same extent as unsorted cells. The karyotypic stability of both sorted and unsorted cells was confirmed, as was their continued pluripotentiality *in vitro*. Interestingly, differentiation into the endoderm lineage, indicated by the expression of α-fetoprotein (AFP), appeared to be achieved more readily with undifferentiated human ES cells that had been sorted, based on forward (FSC) and side (SSC) scatter, for viability, although this could simply be due to chance. Differentiation into the 3 germ layers was not performed using directed differentiation protocols and therefore, it is possible that the wells selected for AFP staining simply did not contain cells of the germ layer which expressed AFP. Alternatively, it is possible that by exposing the cells to sorting, selection of a "juvenile" cell, with increased potentiality has occurred. The directed osteogenic differentiation protocol indicated little difference between the ability of sorted and unsorted human ES cells to produce bone (mesoderm). However, to test the possibility that a "juvenile" cell with increased potentiality had been selected, it would be interesting to direct differentiation of sorted and unsorted human ES cells into cell types of the endoderm lineage, such as hepatocytes.

Human ES cells which had under gone differentiation were however, less amenable to the process of cell sorting. It is appropriate to point out that cells referred to in this chapter as differentiated, have not been directed to differentiate into a particular lineage, and are defined as differentiated based on their non-ES like morphology. Prior to harvesting for cell sorting these differentiating human ES cells had a steady rate of proliferation. It is possible that this proliferative capacity was as a result of association with small populations of undifferentiated human ES cells or progenitor cells, which when grown as monolayers, were in close contact, possibly contained within cell aggregates, to the differentiated cells. It is hypothesised that during cell sorting, the pressurised environment in which the cells were place, encouraged the breakdown of cell aggregates, which would have removed any close association the differentiated ES cells had with undifferentiated ES or progenitor cells. Consequently, when the single cell suspension was plated at low density, the lack of cell-to-cell contact with highly proliferative cells perhaps induced senescence in the differentiated population. If this explanation is correct then plating differentiated cells at higher density on a smaller surface area could improve the level of cell survival but it is unlikely that the proliferative capacity of differentiated cells would improve.

The ability of human ES cells to differentiate into cells representative of the three germ layers holds a great deal of promise for their use in regenerative medicine. However, the majority of the published data describes the potential of populations of human ES cells, which could contain several precursors or stem cells committed to different lineages. Amit et al., (2000) described the first clonally derived human ES

cell lines, providing the first evidence that each human ES cell had the capacity to proliferate indefinitely and to develop into representative cell types of the 3 germ layers. However, the efficiency at which clonal cell lines were derived was very poor, a frequency of just 0.52% (Amit et al., 2000). The preliminary data presented here indicates that undifferentiated human ES cells can be successfully and efficiently single cell cloned using FACS, with a frequency of 10.4%, and that the proliferating cells continued to express Oct-4. However, as previously mentioned, these results represent a single experiment and must be repeated to confirm the observations. It is also essential that these clonal human ES cells are expanded into cell lines and characterised for karyotypic stability and continued pluripotentiality, if this technique is to be used routinely to derive clonal ES cell lines. Furthermore, it would be interesting to determine if the frequency of isolating single cell clones can be increased through culture in low oxygen. Ezashi *et al.,* 2005, have reported that human ES cells grown under conditions of hypoxia (3-5% O_2) undergo less spontaneous differentiation than the same cells cultured under conditions of normoxia (21% O_2). The hypothesis therefore, is that if spontaneous differentiation hampers the ability of human ES cells plated at low density to survive, then perhaps culturing cells in hypoxia may help to over come this. Currently members of the McWhir lab are under taking these experiments.

6.4 Conclusion

As a new piece of equipment the BD FACSAria has been shown to reproducibly and reliably sort differentiated and undifferentiated human ES cells, producing analytical data comparable to that obtained for the BD FACScan. The data presented in this chapter clearly indicate that undifferentiated human ES cells can be successfully sorted using FACS and that the surviving cells continued to grow and behave as undifferentiated human ES cells: expressing characteristic ES cells markers, maintaining a normal karyotype and retaining the ability to differentiate into cells representative of the three germ layers. Furthermore, presented here are preliminary data to support the use of FACS to derive single cell clones, at relatively high frequency (10.4%), with an ES-like morphology and continued expression of Oct-4.

Under the conditions described in this chapter, differentiated human ES cells appeared less amenable to sorting than their undifferentiated counter-parts. Significantly increased levels of cell death were observed when differentiated cells were plated at low density, compared to undifferentiated human ES cells, which consequently reduced their plating efficiency and proliferative capacity. However, reduced cell survival could not be attributed to the process of sorting, since the same effects were observed in the unsorted controls. Therefore, in future experiments the plating density of differentiated cells should be increased in an attempt to improve sustainability of the cultures.

CHAPTER 7 FLUORESCENCE ACTIVATED CELL SORTING (FACS) AS A METHOD FOR SELECTIVE ELIMINATION OF UNDIFFERENTIATED HUMAN ES CELLS.

7.1 Introduction

In Chapter 6, fluorescence activated cell sorting (FACS) was shown to reproducibly sort undifferentiated human ES cells, with no adverse effects on their proliferative capacity. This chapter investigates the possibility of using FACS to deplete undifferentiated human ES cells, with tumorigenic potential, from mixed populations, as an alternative to complement-mediated lysis, reported in Chapters 4 and 5. Endogenous cell surface markers (SSEA-4 and TRA-1-81), which have been reported in the literature to be characteristic of undifferentiated human ES cells

(Reubinoff *et al.,* 2000; Thomson *et al.,* 1998) showing rapid down-regulation with differentiation (Draper *et al.,* 2002), will be used to assess the appropriate regulation of transgenic α-gal expression, with time in differentiation. In addition endogenous cell surface markers indicative of differentiation (SSEA-1 and GD2) will also be assessed in longitudinal studies in an attempt to identify differentiated and undifferentiated cells in a mixed population. SSEA-1 has been discussed elsewhere (see section 1.6.3), its expression is found on undifferentiated mouse ES cells, is absent on undifferentiated human ES cells but shows a transient up-regulation as human ES cells differentiate (Draper *et al.,* 2002). The ganglioseries glyolipid, GD2, is a marker on neuroectoderm and consequently has been shown to have no or very low expression on undifferentiated human EC and ES cells. However, with onset of differentiation GD2 expression is stably upregulated showing a progressive increase in expression with time in differentiation (Draper *et al.,* 2002; Andrews *et al.,* 1990).

Cell sorting experiments were designed to collect both positive and negative cell fractions stained for the expression of either the TRA-1-81 endogenous ES cell epitope or transgenic α-gal. Sorted populations, both negative and positive, were subsequently cultured in conditions known to support the proliferation of undifferentiated human ES cells, should they be present. The results presented in Chapter 6 provide confidence that if contamination of the differentiated population persisted, undifferentiated ES cells would be readily identified.

7.1.1 Chapter Aims

1. To characterise the longitudinal expression of SSEA-4, TRA-1-81 and α-gal as ES specific markers following *in vitro* differentiation.

2. To identify cells positive and negative for endogenous ES or α-gal epitopes in a mixed population of undifferentiated and differentiated human ES cells.

3. To determine the potential of undifferentiated human ES cells to proliferate following time in differentiation medium.

4. To compare endogenous ES cell and α-gal epitopes in their use as ES specific markers for the removal of contaminating undifferentiated ES cells from differentiated populations.

7.2 Results

7.2.1 Expression Profiles of ES Cell Surface Markers are Comparable in Both Transgenic and Wild Type Human ES Cells, with the Exception of SSEA-4.

To use either endogenous cell surface markers or α-gal expression under hTERT control as a method for selectively removing undifferentiated human ES cells from a mixed population, it was first necessary to establish longitudinal characterisation of their expression. To do this, wild type H9 cells and transgenic M2 cells were differentiated over a period of 22 days and assessed for the continued expression of characteristic markers of undifferentiated ES cells (SSEA-4 and TRA-1-81), markers of differentiation (SSEA-1 and GD2) and M2 cells were assessed for expression of transgenic α-gal. Initially, the FSC and SSC profile of undifferentiated H9 and M2 cells was analysed using the BD FACSAria to determine if the process of genetic manipulation had affected the ES cell profile as determined by flow cytometry. Reassuringly, the FSC and SSC profiles of H9 and M2 cells were very similar (Figure 7.2.1), suggesting that the size and complexity of the ES cells had not been affected by genetic engineering and selection.

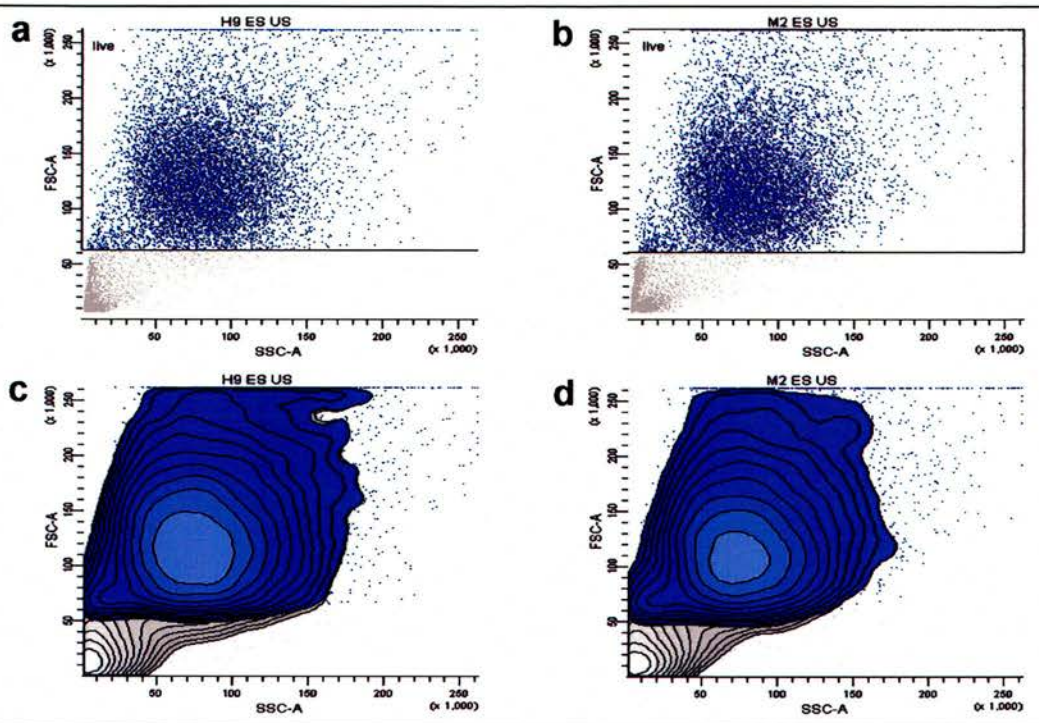


Figure 7.2.1: Transgenic and wild type human ES cells, maintain the same forward (FSC) and side (SSC) scatter profiles. Dot (a and b) and density contour (c and d) plots indicate that the forward (FSC) and side (SSC) scatter of wild type H9 (a and c) and transgenic M2 (b and d) cells remain unchanged, graphs are representative of those observed throughout the investigation and are based on 10,000 counted events within the live gate. Grey represents cells considered to be dead or cellular debris, while blue represents viable cells, referred to as being in the "Live" gate. Contour lines represent a 70% log decrease in cell number.

However, while the expression patterns of SSEA-1, GD2 and TRA-1-81 were comparable between the undifferentiated transgenic M2 cells and the parental cell line (H9), SSEA-4 expression was different (Figure 7.2.2A). H9 cells showed two distinct cell populations, a large negative population, constituting 68.6% of the total, and a minor population positive for SSEA-4 (Figure 7.2.2A H9). M2 cells, on the other hand, which were of H9 origin, only displayed a single positive population for SSEA-4, showing 96.3% positive staining (Figure 7.2.2Aa and Table 7.2.1).

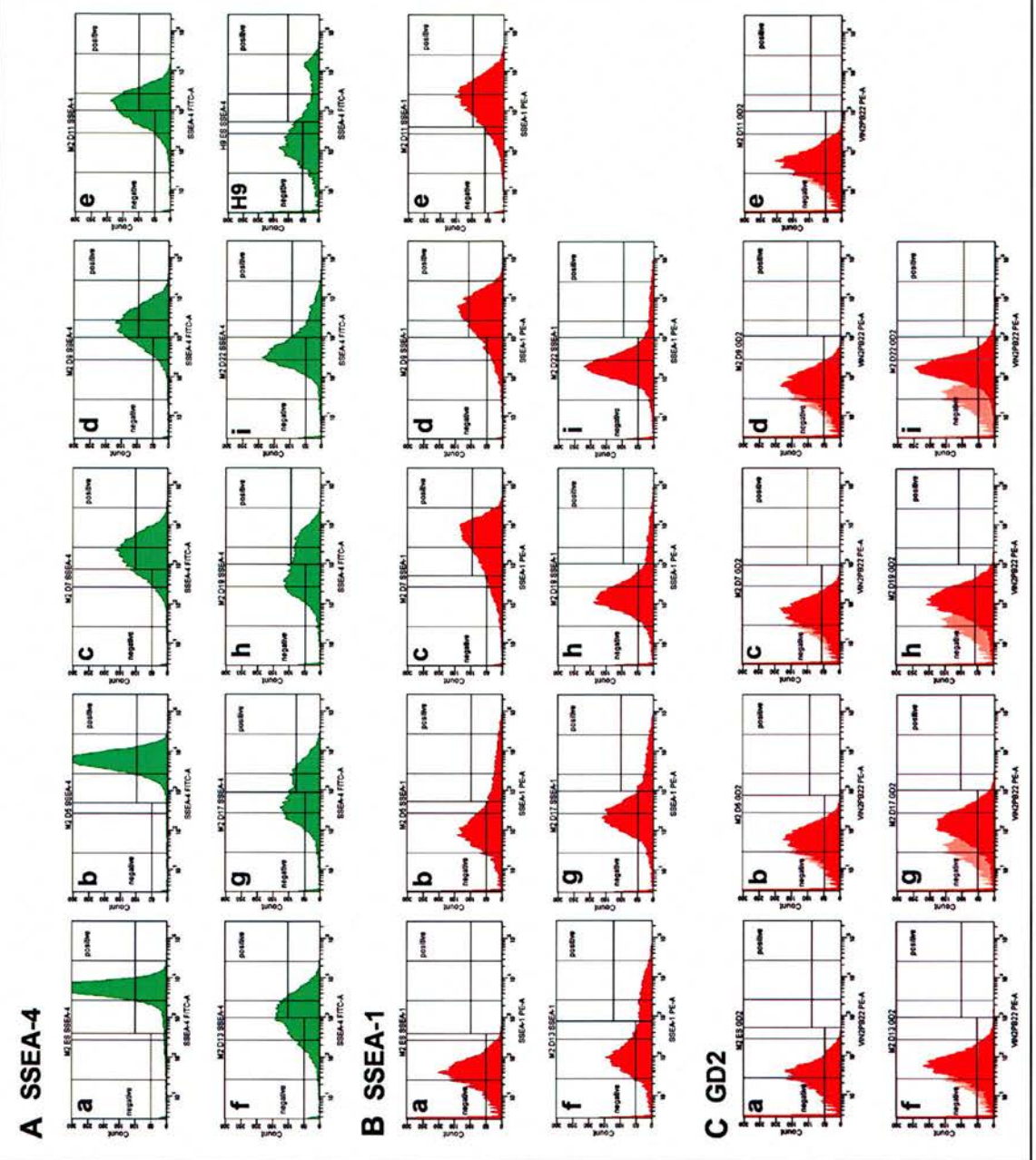


Figure 7.2.2: Longitudinal characterisation of the expression of the cell surface markers SSEA-4 (A), SSEA-1 (B) and GD2 (C), on differentiating M2 human ES cells. Time course for differentiation represented at 0, 5, 7, 9, 11, 13, 17, 19 and 22 days (a-i respectively). Where M2 cells behaved differently to their H9 parental cell line, the H9 histograms are provided (H9). Histograms are representative of three independent experiments and show a minimum acquisition of 10,000 "live" events. The position of the negative gate was derived from time course matched isotype control samples. Data were acquired using the BD FACSAria in collaboration with Martin Waterfall (Roslin Institute, Edinburgh).

M2 Days in differentiation	Proportion (%) of cells positive for the cell surface marker				
	SSEA-4	TRA-1-81	α-gal	SSEA-1	GD2
0	96.3	91.7	98.5	0.5	0.1
5	94.8	94.3	95.6	23.0	0.1
7	74.5	91.9	70.4	84.4	0.1
9	71.3	78.5	71.6	81.2	0.1
11	58.5	66.9	43.8	76.7	0.0
13	51.9	48.9	39.2	25.0	0.2
17	42.2	36.3	27.2	20.0	1.6
19	42.3	22.5	25.8	12.0	0.6
22	26.7	14.1	8.8	11.7	2.9
H9 ES Cells	30.2	92.9	0.6	11.6	0.2

Table 7.2.1: Positive expression of endogenous cell surface markers (SSEA-4 and TRA-1-81) by ES cells decrease with time in differentiation, with a corresponding increase in expression of characteristic markers of differentiation (SSEA-1 and GD2). Expression of α-gal driven by the hTERT promoter follows a similar pattern to SSEA-4 and TRA-1-81 in its down regulation with time in differentiation. Data were acquired on a BD FACSAria in collaboration with Martin Waterfall (Roslin Institute, Edinburgh) and represent 10,000 live events. These data are representative of three independent experiments.

A possible explanation for variation in SSEA-4 expression between the parental H9 cell line and M2 transgenic clonal cell line is that the integration of DNA which led to the establishment of the M2 cell line, integrated into the parental cell line in a cell positive for SSEA-4. However, observations from within the group show that expression of SSEA-4 on the H9 ES cell line is highly variable between different preparations of cells. The H9 cells used for these experiments, although from similar stocks, were not from the same cell preparation that the M2 cell line was derived from. Despite the apparent differences in expression on undifferentiated cells, SSEA-4 was down-regulated in a similar manner on the positive population in both H9 and M2 cells (Figure 7.2.2 shows data for M2 cells only) during the process of differentiation.

SSEA-1 and GD2 are both markers of differentiation. SSEA-1 is reported to be an early onset marker of differentiation, with transient expression, while GD2 is a marker expressed in the later stages of differentiation, after SSEA-1 has switched off again (Draper *et al.,* 2002). A comparison of the expression of both SSEA-1 and GD2 on H9 and M2 cells, indicated no significant difference between the two cell lines, and confirmed that SSEA-1 was a transient marker of early stages of differentiation, being up regulated as early as day 5 (23%) increasing to maximal expression between days 7 and 11 (~80%) before reducing to 25% by day 13 (Figure 7.2.2B and Table 7.2.1). GD2 on the other hand showed only low expression on differentiating human ES cells. Although there was not a significant shift of the peak to the positive gate (only 2.9% of cells after 17-22 days in differentiation) there was gradual shifting of the mean fluorescence intensity with time in differentiation, from a mean fluorescence intensity of 30 to 197 (Figure 7.2.2C and Table 7.2.1) following 17-22 days.

TRA-1-81 is one of a panel of markers used to characterise undifferentiated human ES cells. Expression of TRA-1-81 was found to be consistent on both H9 and M2 cell lines throughout the time-course of differentiation. Down regulation of TRA-1-81 was, however, relatively slow compared with that of SSEA-4, which initial increased remaining positive on over 90% of cells following a week in differentiation medium (Figure 7.2.3A and Table 7.2.1). This was followed by a drop in expression between day 7 and 9 of differentiation reducing the positive population to ~78%. After 2 weeks of differentiation a second more substantial drop in expression was observed resulting in TRA-1-81 expression on only ~50% of the

cells. A gradual decline of TRA-1-81 expression was then observed between 13 and 22 days resulting in just 14.1% of cells remaining positive compared to 26.7% of cells expressing SSEA-4 at the same point of differentiation (Table 7.2.1 and Figure 7.2.3A).

To determine the potential for hTERT driven expression of α-gal epitopes to selectively remove undifferentiated cells, instead of endogenous ES cell markers, their expression was also characterised over time in differentiation. As expected M2 cells expressed high levels of α-gal in their undifferentiated state (Figure 7.2.3Ba 98.5%) while the H9 parental cell line was essentially negative (showing 0.6% positive staining Figure 7.2.3B-H9 probably caused by no-specific binding of the *BS-IB$_4$* lectin). As with SSEA-4, down regulation of α-gal was observed immediately after the induction of differentiation, and occurred at a similar rate. Expression of α-gal had reduced to 71.6% of the cells by 7 days, compared to 74.5% of cells that expressed SSEA-4 at the same time point while over 90% of cells continued to express TRA-1-81. Following 9 and 11 days of differentiation, α-gal expression was reduced to approximately 40% of the population. This down-regulation of α-gal expression continued with time in differentiation and after 22 days, only 8.8% of the population continued to express α-gal, compared to 26.7% expressing SSEA-4 and 14.1% expressing TRA-1-81 (Figure 7.2.3B, Figure 7.2.2A, Figure 7.2.3A respectively and Table 7.2.1.). Interestingly this was a similar level to the percentage Calcein-release that was observed after M2 cells, differentiated for 21 days, were exposed to human serum (8.8% Figure 5.2.8), suggesting a residual α-gal expressing population.

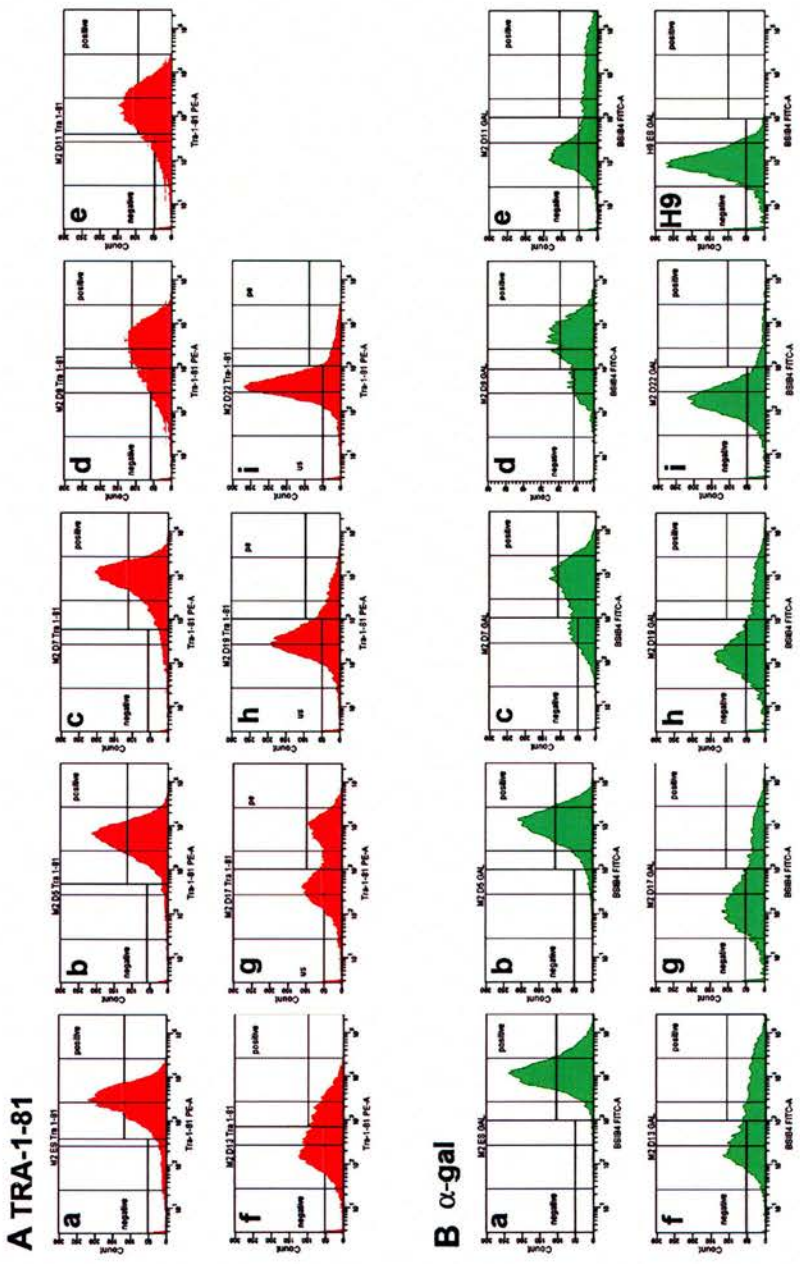


Figure 7.2.3: Longitudinal characterisation of the expression of the cell surface markers TRA-1-81 (A) and α-gal (B) on differentiating M2 human ES cells. Time course for differentiation represented at 0, 5, 7, 9, 11, 13, 17, 19 and 22 days (a-i respectively). Where M2 cells behaved differently to their H9 parental cell line, the H9 histograms are provided (H9). Histograms are representative of three independent experiments and show a minimum acquisition of 10,000 "live" events and the position of the negative gate was derived from time course matched isotype control samples. Data were acquired using the BD FACSAria in collaboration with Martin Waterfall (Roslin Institute, Edinburgh).

7.2.2 Nine Days in Differentiation Medium Significantly Reduces the Ability of Human ES Cell Derivatives to Revert Back to Their Undifferentiated State.

In an attempt to identify a "natural" population, which contained both undifferentiated and differentiated derivatives of human ES cells, H9 cells were differentiated using basic differentiation medium, but instead of making EBs, cells were differentiated as monolayers, plated on matrigel coated plastic. The cells were maintained, in triplicate wells, in differentiation medium for 3, 6, 9, 12, 15 and 18 days and showed clear changes in morphology (Figure 7.2.4).

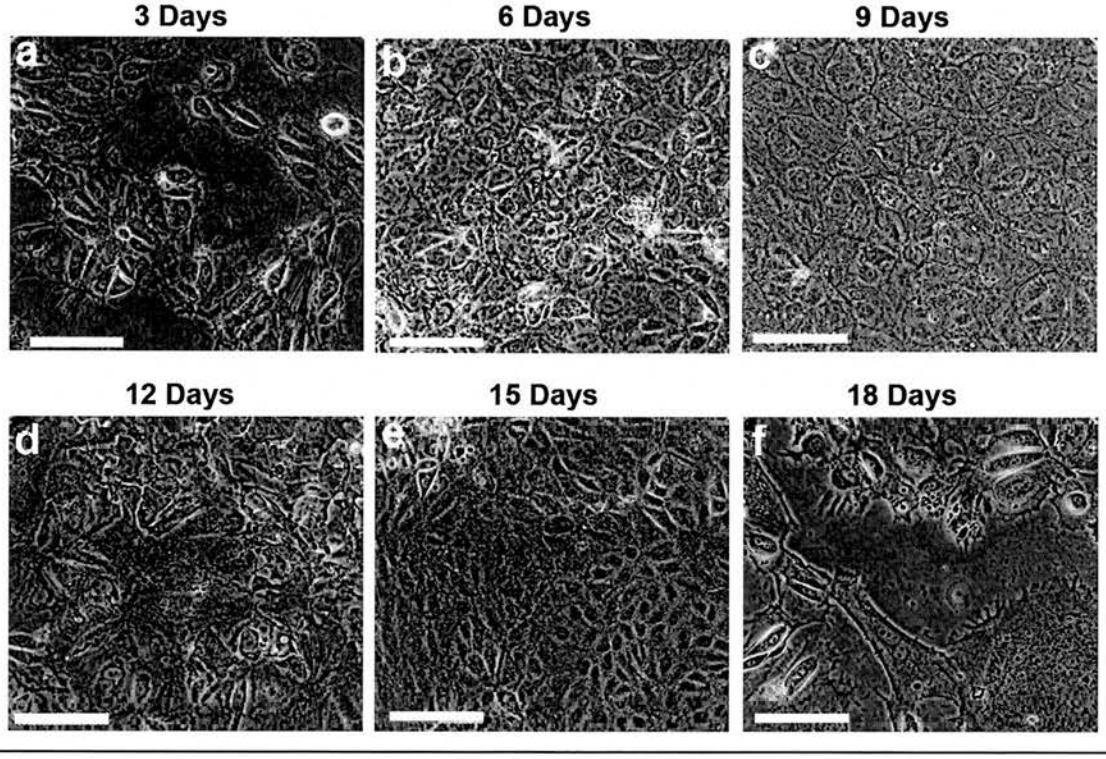


Figure 7.2.4: Human ES cells were differentated as monolayers in basic differentiation medium and assessed for changes in cell morphology. The time course of differentiation was 3, 6, 9, 12, 15 and 18 days (a-f respectively). Images are representative of triplicate wells. Scale bars represent 100μm.

At each of these differentiation time points the cells were passaged (1 in 2) and were placed back into culture conditions that favoured growth of undifferentiated ES cells, in conditioned medium supplemented with hbFGF (Figure 7.2.5). In every case, when cells had been in differentiation medium for only 3 days, human ES cells emerged at high frequency, and also proliferated well (Figure 7.2.5 a and b).

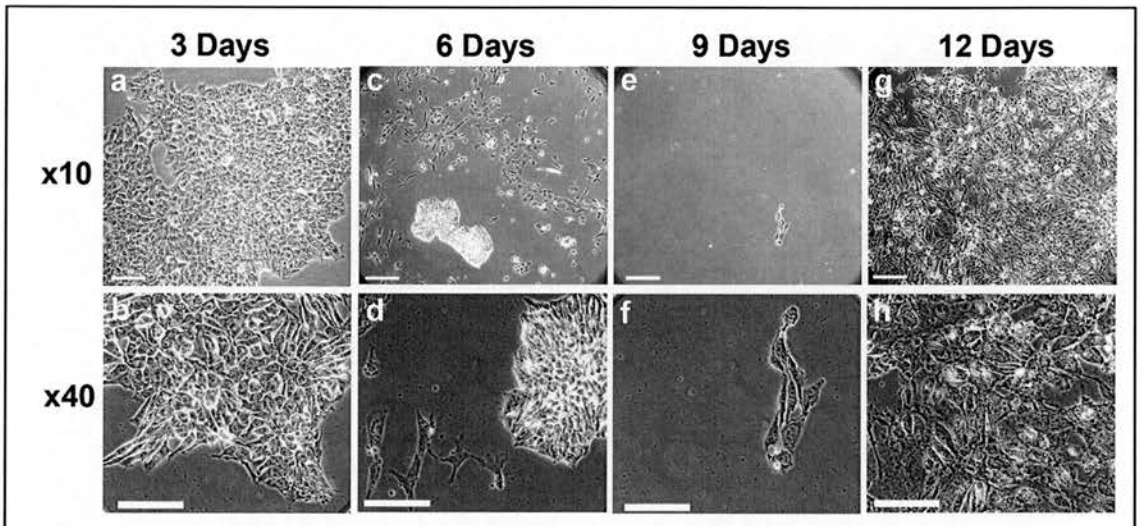


Figure 7.2.5: Human ES cell-derivatives placed back into ES culture conditions (conditioned medium plus hbFGF) following 3, 6 and 9 days in differentiation medium (a&b, c&d and e&f respectively) give rise to ES-like cells. By 9 days the surviving ES-like cells were few and in most cases they didn't proliferate well. Following 12 days of differentiation, cell survival at passage significantly improved, leading to the presence of more cells in undifferentiated ES cell conditions (g&h). However, these cells were not observed to proliferate, they did not have an ES-like morphology, and gradually they declined in number during the 14 days of undifferentiated culture. Images are representative of triplicates, from duplicate experiments. Scale bars represent 100μm.

Human ES like-cells also appeared, at increasingly lower frequencies, when cells had been exposed to differentiation medium for 6 and 9 days (Figure 7.2.5 c and d,

and e and f respectively). However, when human ES cells were differentiated for 12 days before being placed back into conditions for undifferentiated ES cell culture, there was an unexpected increase in the number of cells that survived passage which had not be seen at earlier time points. The reason for this increase in survival may have been due to cell expansion with time in culture. At each time point, cells were not counted prior to passage, they were split 1 in 2, with half of the cells being placed into conditions for undifferentiated ES cell culture. Perhaps a more stringent experimental design would have been to count cells prior to passage, maintaining a constant seeding density. However, despite this increase in cell number, no cells with ES-like morphology were observed in 12-day differentiated ES cells, following 10-14 days in culture conditions which would favour their growth (Figure 7.2.5 g and h).

This lack of ES-like cell re-growth, post 9 days in differentiation medium, corresponded with the significant increase in SSEA-1 expression and down regulation of SSEA-4, TRA-1-81 and α-gal expression observed by flow cytometry (Figure 7.2.2A and Figure 7.2.3 A and B). Therefore, mixed populations containing differentiated and undifferentiated human ES cells with growth potential were likely to occur between 6 and 9 days in differentiation medium and consequently were used as the start population to evaluate the potential of cell sorting.

7.2.3 Mixed Populations of Differentiated and Undifferentiated Human ES Cells can be Sorted Using FACS

The use of cell sorting to remove minority populations of undifferentiated human ES cells from a population of differentiated derivatives was investigated as an alternative to complement-mediated lysis. Since the expression of SSEA-4 was observed to be variable between different preparations of H9 cells and as there is evidence to show that SSEA-3/4 negative human EC cells retain the ability to form teratomas *in vivo* (Andrews *et al.,* 1985), the decision was taken to use TRA-1-81 as an endogenous cell surface marker, and compare it with the use of transgenic α-gal epitopes, under the control of the hTERT promoter, on M2 cells.

M2 cells were differentiated for either 6 or 8 days as monolayers, in basic differentiation medium. Undifferentiated M2 cells, stained either with *BS-IB$_4$* lectin or Tra-1-81 antibody (Figure 7.2.6 b and e respectively), were used as positive controls to determine the position of the positive sort gates. As negative controls, H9 cells, labelled with *BS-IB$_4$*, were used for α-gal sorts (Figure 7.2.6a) and M2 cells, labelled with an IgM isotype control antibody, were used as the negative control for TRA-1-81 sorts (Figure 7.2.6d). These control populations were used to define the upper limits of the negative sorting gates. To reduce contamination of the sorted populations as a result of conflicts around the upper and lower limits of the sort gates, an area between the upper boundary of the negative gate and the lower boundary of the positive gate was excluded for this sort strategy (Figure 7.2.6 c and f).

Once the sort gates had been defined, the sort was designed to collect a minimum of $2x10^6$ cells from the negative and positive gates, discarding those cells that fell between the two gates. Cells were collected in 5ml polystyrene tubes (Falcon) containing 1ml of conditioned medium (positive sort) or 1ml of basic differentiation medium (negative sort) supplemented with 100U penicillin and 100µg streptomycin, to reduce the possibility of opportunistic infection during the sorting process.

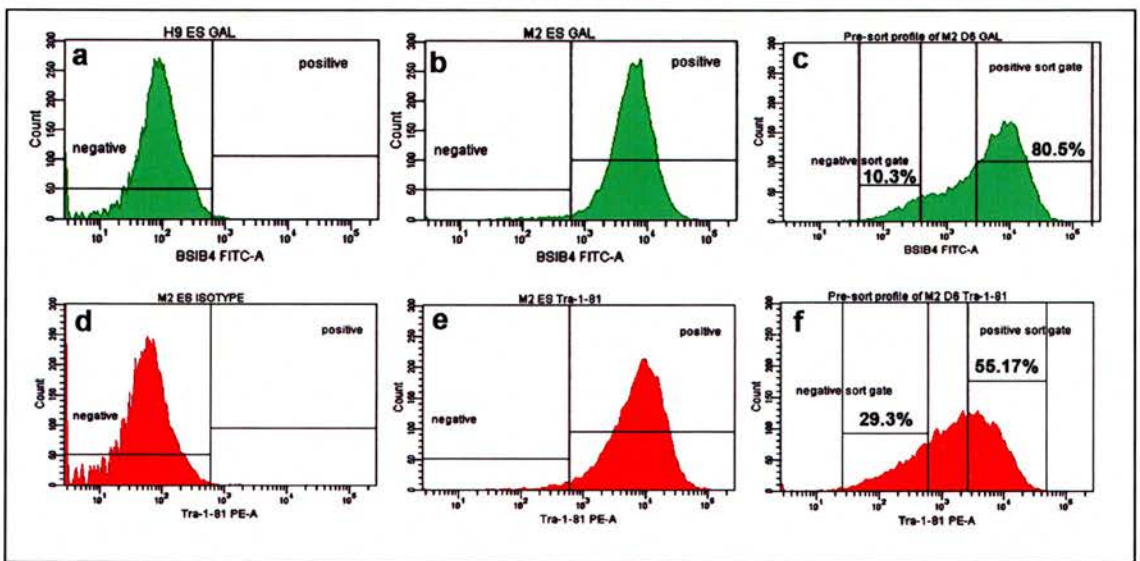


Figure 7.2.6: Sort profiles were established for both α-gal (a-c) and TRA-1-81 (d-f) sorting, using negative (a and d) and positive (b and e) controls to determine the sort gates (c and f). To reduce conflict around the upper and lower boundaries of the negative and positive sort gates (respectively) an area between the two was left un-gated, referred to as the conflict zone. Histograms represent 10,000 events. Data was acquired using the BD FACSAria in collaboration with Martin Waterfall (Roslin Institute, Edinburgh).

After sorting, the collected cell populations were washed to remove traces of sheath fluid (sterile PBS) and cellular debris, and a small sample of 2-5,000 cells was reacquired to assess the purity of the sorts. Analysis of the post-sort profiles

indicated that the sort had been successful: taking into account that the start population was a mix of cells with indiscriminate expression of the respective markers. Reacquisition data indicated a 9- and 3-fold enrichment of the negative, potentially differentiated, population from 10.3% and 29.3% to 97.3 and 91.5% for α-gal and TRA-1-81 sorts respectively (Figure 7.2.7). Similarly, positive sorts for α-gal and TRA-1-81 indicated enrichment in the population of 8% and 39.8% respectively. However, it is possible that this is an underestimate of enrichment, since quenching of the fluorochrome and re-distribution of the live events about a new mean, has resulted in a slight shift in the histogram peaks towards the negative upon reacquisition of the data (Figure 7.2.7 c and f).

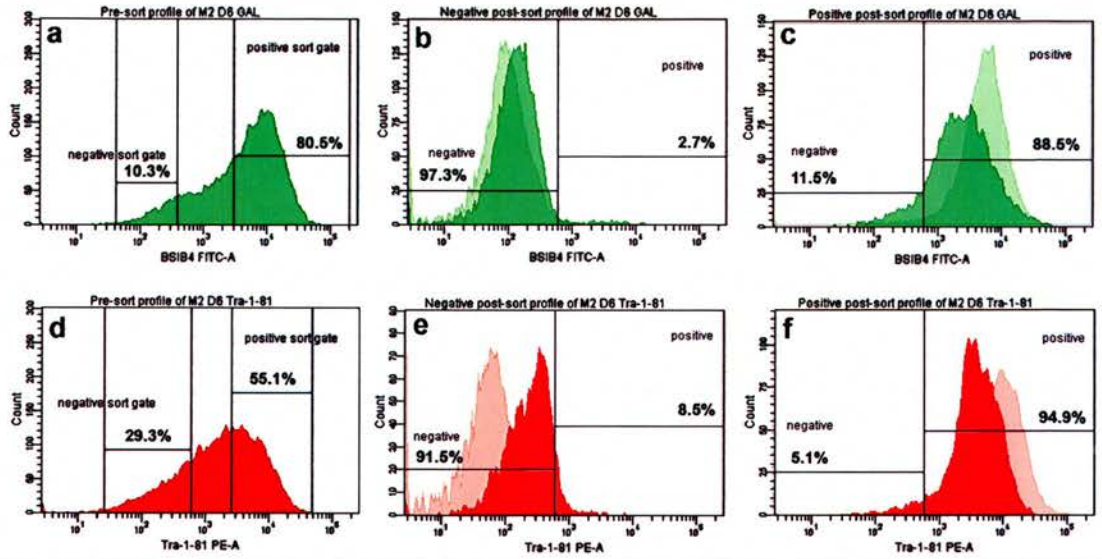


Figure 7.2.7: Pre-sort and post-sort profiles of human ES cells differentiated for 6 days in basic differentiation medium and sorted based on their continued expression of either α-gal (a-c) or TRA-1-81 (d-f). Histograms a and d show the mixed starting population stained for either α-gal (a) for TRA-1-81 (d), b and e show the post-sort profiles of the two negative populations and c and f show the post sort profile of the positive populations α-gal and TRA-1-81 (c and f respectively). Shaded histograms represent the position of the respective negative (b and e) and positive (c and f) populations used to define the sort gates. Histograms for post-sort profiles represent between 2,000 and 5,000 events only, while pre-sort histograms represent 10,000 events, the axes have been adjust accordingly. Post sort gates were defined from the staining profiles of H9 ES (negative control) and M2 ES (positive control) with BS-IB4 or Tra-1-81 and relevant isotype control as indicated in Figure 7.2.6 a&b and d&e respectively. Data acquired using a BD FACSAria in collaboration with Martin Waterfall (Roslin Institute, Edinburgh).

In terms of the elimination of undifferentiated human ES cells from within a mixed population it was necessary to determine whether this percentage enrichment of the negative population corresponded to reduced contamination by undifferentiated human ES cells. Consequently, after sorting cells, both the positive and negative fractions were re-plated in triplicate wells of a 12-well plate, at a density of $1x10^5$ per well, in both undifferentiated and differentiation culture conditions. As controls,

undifferentiated human ES cells were plated in triplicate at densities of $1x10^5$ and $1x10^2$; $1x10^2$ undifferentiated ES cells/well would be the equivalent of a 0.1% contamination in a mixed population. These wells would therefore demonstrate that this level of contamination could be detected in $1x10^5$-sorted cells, should they be present. The mixed "unsorted" population was also plated in triplicate at a density of $1x10^5$, to determine the extent of surviving undifferentiated human ES cells, after 6 days in differentiation medium.

Cell viability was determined using Acridine Orange/Ethidium Bromide staining, following 7-10 days in culture. Acridine Orange (AO) permeates all cells and makes the nuclei appear green, while Ethidium Bromide (EtBr) is excluded from living cells, only being incorporated into cells that have lost cytoplasmic membrane integrity, staining the nucleus orange/red. In addition AO/EtBr staining can also be used to identify cells that are undergoing apoptosis as opposed to necrosis. Early stage apoptosis is characterised by highly condensed chromatin that is uniformly stained by the AO (appearing as bright green spherical beads) and possibly EtBr uptake in the cytoplasm as a result of disruption to the cell membrane. Late apoptotic cells display condensed and fragmented orange chromatin, again appearing as spherical beads as apposed to the orange structured nuclei observed in necrotic cells.

Undifferentiated human ES cells, seeded at a density of 10^5 per well, became confluent within 2-3 days in medium supporting undifferentiated ES cell growth, producing a sheet of cells that was too dense to use as a control a later time points

(data not shown). However, the same cells plated at 10^2, to represent 0.1% residual ES cell contamination, produced several large colonies following 7-10 days in culture, which stained bright green in the presence of AO/EtBr (Figure 7.2.8a and d).

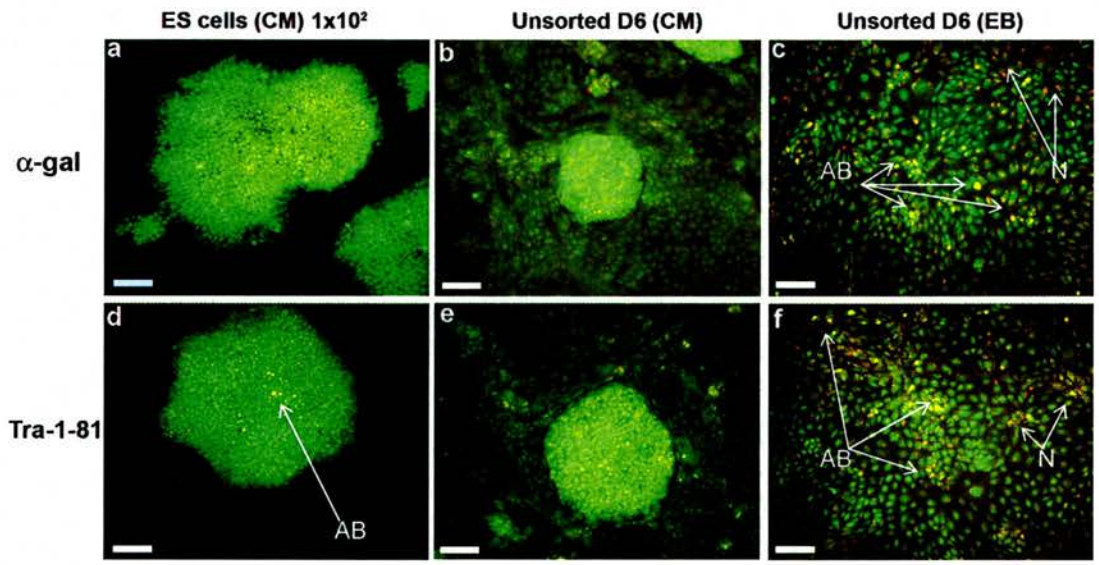


Figure 7.2.8: Acridine Orange/Ethidium Bromide staining of the control populations for the α-gal (a-c) or TRA-1-81 (d-f) sorted. Unsorted undifferentiated (a and d) or partially differentiated for 6 days (b-c and e-f) human ES cells. A similar frequency of undifferentiated human ES cells, was observed in the partially differentiated (6 days (D6)) start population, as was seen in the wells containing $1x10^2$ ES cells (representing a 0.1% contamination) when seeded back into culture conditions which favoured the proliferation of undifferentiated human ES cells. No ES-like colony cells were observed when cells were grown in differentiation medium (c and f). Scale bars represent 100μM. CM = conditioned medium which favours undifferentiated growth, EB = Embryoid Body medium referred to as basic differentiation medium, AB = Late stage apoptotic body and N = necrotic cells.

A similar frequency of undifferentiated human ES cells, was also observed in the partially differentiated (6 days (D6)) start population when seeded back into culture

conditions which favoured the proliferation of undifferentiated human ES cells (Figure 7.2.8 b and e). As expected, cells with ES-like morphology were not observed in the 6-day differentiated start population when placed back into differentiation medium (13-16 days total differentiation Figure 7.2.8 c and f).

By contrast to the results in Chapter 6, differentiated human ES cells adhered well at a density of 10^5, with less cell death; however, although there was increased survival the cells did not proliferate well, never reaching confluence. This reduced proliferation of differentiated human ES cells compared to undifferentiated ES cells was also observed in the uptake of AO/EtBr. AO/EtBr both intercalate into DNA, and therefore, uptake of this stain is dependent on the condensation status of DNA. Highly proliferative cells (undifferentiated ES cells for example) have relaxed DNA, which will readily incorporate AO, producing the bright green stain observed in Figure 7.2.8a, b, d and e. Senescent or quiescent cells, on the other hand are likely to have more condensed DNA, which is unable to incorporate AO as efficiently, leading to a dull green stain (Figure 7.2.8 c and f). Furthermore, in the differentiated cultures there was increased evidence of apoptotic and necrotic cells.

When assessed for the expression of α-gal (*BS-IB$_4$*) and TRA-1-81 undifferentiated controls stained strongly positive (Figure 7.2.9 a and e). The mixed populations placed back into undifferentiated culture conditions stained positive only in areas that either had ES-like morphology or on cells that were adjacent to such colonies (Figure 7.2.9 images b-c and f-g respectively). However, there was evidence of persistent positive staining for α-gal and TRA-1-81 on unsorted cells placed back

into differentiation (EB) medium (total of 13-16 days Figure 7.2.9 e and h respectively). This level of expression was comparable to that observed in the longitudinal expression studies of these markers (Figure 7.2.3).

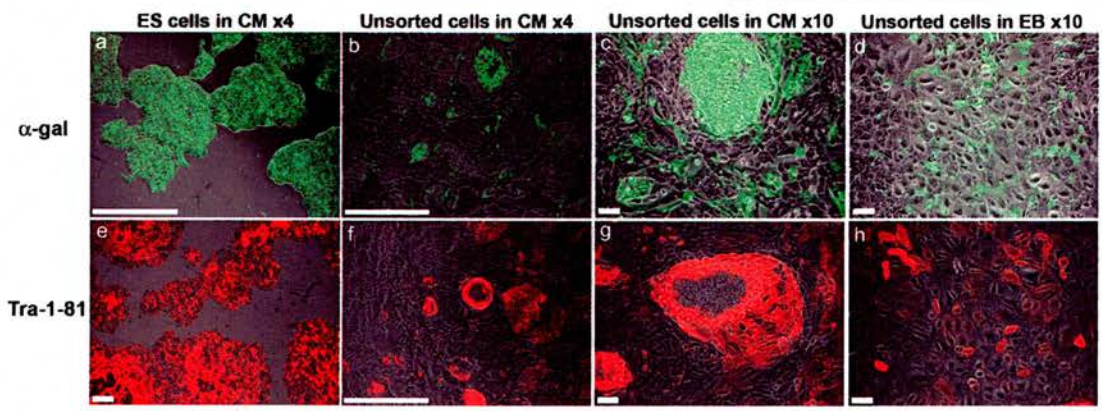


Figure 7.2.9: *BS-IB$_4$* (a-d) and Tra-1-81 (e-h) staining of the unsorted control populations from the α-gal and TRA-1-81 sorts indicated that the persistence of the epitopes was mainly associated with cells of ES-like morphology or in areas that close to ES-like colonies, following 7-10 days of culture. Images are representative of triplicate wells. Scale bars represent 100μm. CM = conditioned medium which favours undifferentiated growth and EB = Embryoid Body medium referred to as basic differentiation medium.

As expected, populations of cells sorted for the positive expression of either α-gal or TRA-1-81 epitopes were found to contain a high incidence of human ES-like cells (Figure 7.2.10 a and d respectively). However, despite the high seeding density of positively sorted cells (10^5/well), the frequency of ES-like cells was more comparable to that observed when unsorted control ES cells were seeded as 10^2. This suggested that partially differentiated cells may have expressed "undifferentiated" markers at the time of the sort, but did not maintain undifferentiated ES cell growth potential. After 7-days in medium which favours

the growth of undifferentiated human ES cells, those ES-like cells that did arise stained strongly for either *BS-IB*$_4$ or Tra-1-81 respectively (Figure 7.2.10 b-c and e-f respectively), confirming that the sorting process did not affect ES cell survival and proliferation.

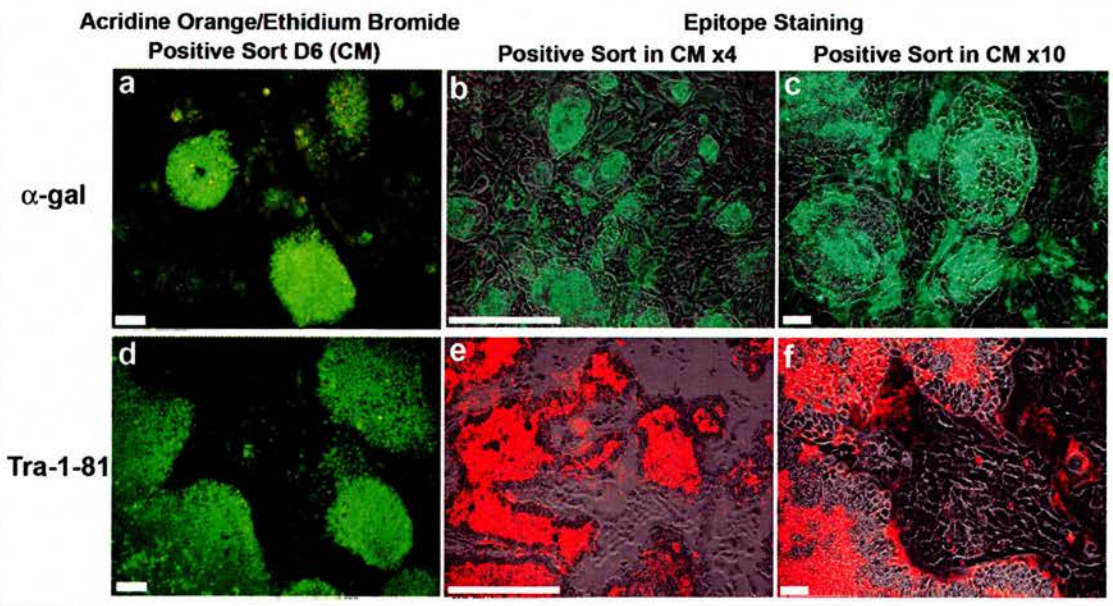


Figure 7.2.10: Partially differentiated (for 6 days) cell populations sorted for the positive expression of either α-gal (a-c) or TRA-1-81 (d-f) showed an abundance of viable cells will ES-like morphology by Acridine Orange/Ethidium Bromide staining when placed back into to conditions that support undifferentiated ES cell growth for 7-10 days (a and d respectively). Furthermore, these cells were highly proliferative and stained positive for α-gal (b & c) and TRA-1-81 (e & f) respectively. Images are representative of triplicate wells. Scale bars represent 100μm. CM = conditioned medium which favours growth of undifferentiated cells.

Interestingly, the frequency of ES-like cells, collected in the population that was positive for α-gal, appeared to be less than in the TRA-1-81 population (Figure 7.2.10 a compared to d) suggesting that differentiated cells with residual α-gal

expression had been included in the positive population, diluting the presence of ES-like cells. During acquisition of the α-gal sort, the *BS-IB$_4$* stain for α-gal appeared to be quenched to a greater extent than the TRA-1-81 stain (Figure 7.2.7 c and f respectively). It was hypothesised that *BS-IB$_4$* lectin may have been sub-optimally bound by partially differentiated human ES cells, which had started to down regulate α-gal epitopes on the cell surface, which was then lost during the sorting process.

Of greater significance however, to this investigation was the emergence of ES-like cells in the negative populations. Following 7-10 days in culture conditions which favour the growth of undifferentiated ES cells, an abundance of viable cells, with ES-like morphology, were observed by AO/EtBr staining in the negative TRA-1-81 population (Figure 7.2.11e) at a frequency comparable to that observed in the cell population sorted as positive for TRA-1-81 expression (Figure 7.2.11e compared to Figure 7.2.10d).

Initially it was considered that contamination of the negative population, as indicated by 8.5% TRA-1-81 positive cells might have accounted for the observed presence of ES-like cells (Figure 7.2.7e). However, as there was a significant increase, in excess of 10-fold, in the number of TRA-1-81 positive cells (Figure 7.2.7f), it was unexpected that similar levels of ES-like cell growth would have been observed in the two populations. These preliminary data strongly suggest that, contrary to current belief, loss of TRA-1-81 does not necessarily indicate terminal differentiation of human ES cells. In addition, TRA-1-81 expression, as detected by immunochemistry with Tra-1-81 antibody, was strongly up-regulated in the

"negative" population following 7-10 days in culture conditions that favour undifferentiated ES cell growth (Figure 7.2.11 g and h).

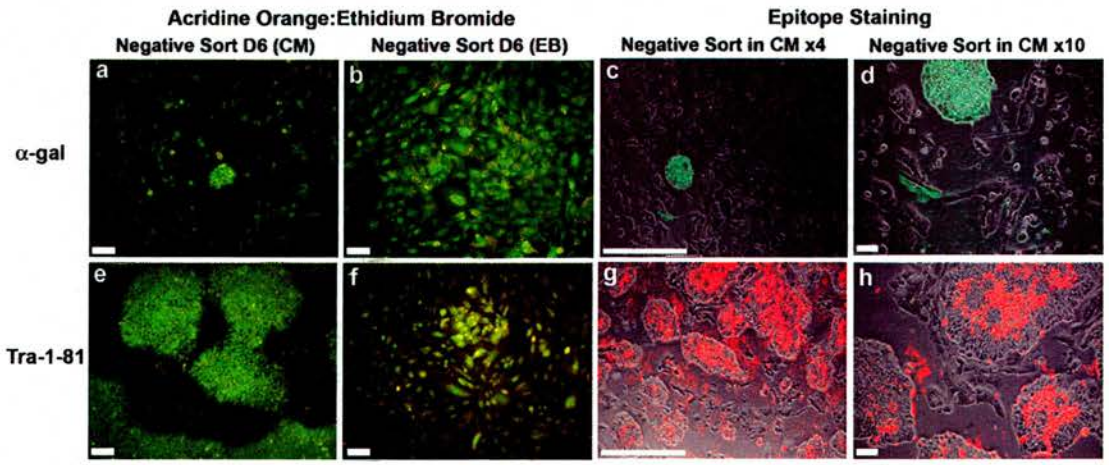


Figure 7.2.11: Partially differentiated (6 days) cell populations were sorted for negative expression of either α-gal (a-d) or TRA-1-81 (e-h) and were subsequently placed back into to conditions that support growth of undifferentiated ES cells (CM). After 7-10 days, the surviving cells were stained with Acridine Orange/Ethidium Bromide to identify viable cells. Under these conditions, small ES-like colonies were occasionally observed in the α-gal negative sort population (a). However, a significant increase in the presence of ES-like cells was observed in the negative TRA-1-81 sort population (e), which showed comparable ES-like cell levels to the positive sort population (Figure 7.2.10d). Furthermore, staining for α-gal (c & d) and TRA-1-81 (g & h) indicated that the persistence of the epitopes was mainly associated with these highly proliferative cells of ES-like morphology or in areas close to ES-like colonies. No ES-like colony cells were observed when cells were grown in differentiation (EB) medium (images b and f). Images are representative of triplicate wells. Scale bars represent 100μm.

By contrast, the α-gal sort demonstrated only an occasional ES-like colony (Figure 7.2.11a), which may have developed as a consequence of contamination by low expressing positive cells in the negative population (Figure 7.2.7b indicates 2.7% contamination) or possibly as a result of de-differentiation, resulting in up-regulation

of hTERT and consequent expression of α-gal. *BS-IB$_4$* staining in the negative α-gal sorted population was specifically restricted to those cells with ES-like morphology, which suggests re-occurrence of expression rather than persistence.

Together these data might imply that down-regulation of α-gal, which lags behind down-regulation of hTERT gene expression, more accurately characterises terminal differentiation, as compared to TRA-1-81.

7.3 Discussion

The preliminary experiments reported in this chapter were designed as a "proof of principle" to determine whether fluorescence activated cell sorting (FACS) could be used efficiently as an alternative to complement-mediated lysis to eliminate undifferentiated human ES cells from a mixed population. In order to achieve this, "naturally" occurring differentiated populations, which had been demonstrated to contain detectable levels of undifferentiated human ES cells with growth potential, were assessed for the expression of cell surface epitopes that could be used to identify contaminating undifferentiated ES cells.

Longitudinal flow cytometry studies demonstrated that the expression of characteristic ES cell surface markers, SSEA-4 and TRA-1-81, showed a steady decline in expression with time in differentiation, a pattern that was also reflected in α-gal expression under the control of hTERT on M2 cells. A significant reduction in the expression of all cell surface markers studied (SSEA-4, TRA-1-81 and α-gal) and up-regulation of the early differentiation marker SSEA-1 was observed between 5 and 9 days of differentiation. This change in expression of cell surface antigens corresponded to a significant reduction in the ability of differentiated populations, at the same time points, to give rise to ES-like colonies when placed back into culture conditions which should favour growth of undifferentiated ES cells, suggesting that perhaps a significant event, such as cell commitment, was taking place. Draper *et al.,* (2002) reported that the surface antigens of the H7 human ES cell line changed upon differentiation. In this report the authors indicated that SSEA-4 and TRA-1-81 both progressively down-regulated with time in "un-inducted" differentiation (i.e. on

matrigel but in the absence of conditioned medium and hbFGF), although it was to a lesser extent than with retinoic acid induced differentiation. However, they indicated that after 9 days of differentiation, H7 cells had down-regulated significantly more TRA-1-81 than SSEA-4 at the same time point, and that in fact SSEA-4 expression initially increased following induction of differentiation before beginning to down regulate, which is the inverse of what was observed in this investigation. Furthermore, Draper *et al.,* indicated very little increase in SSEA-1 expression, unless retinoic acid was used to induce differentiation, yet they show that GD2 expression was almost immediately up regulated without induction, again the reverse of what was seen in the experiments reported here (Draper *et al.,* 2002). It is unclear from the report whether a longitudinal study of the expression of these markers was performed or whether the authors picked the time points at which to assess expression. Interestingly, the time points at which they chose to report were 9 and 16 days. Following 9 days of differentiation Draper *et al.,* reported that a significant number of the markers used to characterise undifferentiated human ES cells (SSEA-3 and -4, TRA-1-60 and -1-81 and also alkaline phosphatase) had all begun to be down-regulated, which is reflected in the experiments reported here. It is likely that the specific differences observed in patterns of down regulation between the Draper *et al.,* (2002) report and this one, are related to the use of different human ES cell lines, and it would be interesting to repeat the experiments reported here to test this hypothesis.

Day 6-9 differentiated populations were demonstrated to produce ES-like cells when placed back into culture conditions for undifferentiated ES cells. Furthermore,

cultures in this period of differentiation were also shown to have begun down-regulation of the characteristic surface markers of undifferentiated ES cells. Therefore, these populations were selected as a target start population to represent a differentiated population contaminated with undifferentiated ES cells which had continued growth potential. It was not possible to detect the appearance of undifferentiated ES cells when 8 day differentiated ES cells were sorted and returned to undifferentiated culture conditions (data not shown). Consequently all subsequent sorting experiments therefore used human ES cells differentiated for 6 days.

A limitation of this experimental design was that after only 6 days in differentiation medium definitive discrimination of cells that were positive for continued expression of the desired epitopes (α-gal and TRA-1-81) from those which were truly negative was not possible; a large proportion of the start population had varying degrees of epitope expression at this time point. Differentiation of ES cells for 6 days may not reflect the period from which therapeutic cells would be isolated. To use time points later in differentiation would have simplified the cell sorting strategy, by providing clearer distinction between negative and positive cells, however in order to evaluate the success of FACS at eliminating undifferentiated ES cells, the start population had to contain significant levels of cells with undifferentiated *in vitro* growth potential to allow for post sort analysis.

Despite starting with an indiscriminately stained population, cell sorting based on the positive and negative expression of either α-gal or TRA-1-81 was very

successful, with negatively expressing populations being enriched from 10.3% and 29.3% to 97.3% and 91.5% respectively. However, while enrichment of a population is a good measure of the success of FACS, only re-culturing of the sorted populations could demonstrate the efficiency of the selection marker(s) to identify contaminating cells with undifferentiated growth potential. In the report by Draper *et al.,* (2002), the authors did not attempt to re-culture the "differentiated" cells that had reduced expression of "characteristic ES markers" in conditions that favour undifferentiated ES cell growth to determine if loss of characteristic markers of undifferentiated ES cells was indicative of differentiation, their assessment was made on changes in morphology only.

Positive and negative sort populations were seeded back into to culture conditions that favoured the growth of undifferentiated ES cells and were assessed for the presence of viable cells with ES-like morphology and growth potential. In both α-gal and TRA-1-81 positive sort populations there were significantly fewer ES-like colonies than in the unsorted, undifferentiated ES cell controls, seeded at the same density (10^5) (data not shown). The frequency of ES-like colonies in the positive TRA-1-81 sort was similar to that observed in the unsorted undifferentiated ES cells seeded at 10^2, suggesting that some of the TRA-1-81 positive cells were partially differentiated. Furthermore, in the α-gal sort the frequency of ES-like cells appeared to be less than in the TRA-1-81 population, suggesting that either differentiated cells maintained low level α-gal expression on their cell surface, or that the *BS-IB$_4$* lectin was sub-optimally binding to partially differentiated human ES cells that had started to down regulate α-gal epitopes on the cell surface.

The *BS-IB$_4$* lectin has four binding sites by which it can bind to α-gal epitopes, however, for stable binding the lectin must be bound by at least two of these sites (Galili 1999a). Therefore, negative staining by *BS-IB$_4$* lectin may not necessarily mean a lack of α-gal epitopes, just that the epitopes are to far apart for the lectin to bridge the gap and bind to at least two. It has been reported that binding via just one of the lectins binding sites is too weak to sustain stable binding and so the lectin is easily removed by washing (Galili 1999a). This explanation corresponds with the staining pattern that was observed for the mixed population used for the α-gal sort. Initially, when the sort gates were defined, immediately after staining, the population stained strongly with *BS-IB$_4$*. During the course of cell sorting, under constant pressure and continuous mixing of the cell suspension, the *BS-IB$_4$* staining became weaker, suggesting that loosely bound lectin was being removed. If this was the case it would help to explain why the frequency of ES-like cells in the positive α-gal population was lower than in the positive TRA-1-81 population. Weak binding of *BS-IB$_4$* to a single α-gal epitope could have resulted in the inclusion of α-gal positive cells, which were actually partially differentiated, consequently diluting the number of high level expressing, potentially undifferentiated ES cells.

The removal of positive, potentially tumorigenic cells from the negative, potentially therapeutic populations was of greater importance in the experimental design of this thesis. Negative populations, cultured for 7-10 days in medium that favours undifferentiated ES cell growth, which had been enriched for loss of TRA-1-81

expression were shown to contain an abundance of ES-like cells, strongly suggesting that loss of TRA-1-81 was not indicative of a reduction in undifferentiated ES growth potential. A comparison of the negative (91.5% cells) and positive (94.2% cells) populations by fluorescence microscopy of acridine orange/ethidium bromide (AO/EtBr) stained cells indicated a similar level of ES-like cell growth in both populations, strongly suggesting that, contrary to current dogma, TRA-1-81 may be a poor marker of ES cell differentiation.

By comparison, expression of α-gal under the control of the hTERT promoter appeared to be significantly better at discriminating between the differentiation status of ES cells. The negative α-gal population contained far fewer undifferentiated ES-like cells compared to the TRA-1-81 negative population. Furthermore, ES-like colonies that did appear in the α-gal negative population were much smaller, suggesting that they might have arisen during the time in culture, or that as low density contaminants from the sort process the ES cells had experienced delayed proliferation due to their isolation from neighbouring ES cells (Amit *et al.,* 2000; Thomson *et al.,* 1995). There are a number of possible explanations for the persistence of ES cells, with undifferentiated growth potential. As shown in Chapter 3, expression of α-gal on M2 cells was affected by position effect variegation, so it is possible that evading cells were not expressing α-gal at the time cell sorting took place. Alternatively, it is possible that the α-gal negative sort gate was too generous, to collect sufficient cells for analysis, which consequently allowed only partially differentiated cells with reduced α-gal expression to be included in the negative sort. Alternatively, it is possible that cell sorting did eliminate all α-gal expressing

undifferentiated cells, but that during the re-culture, partially differentiated cells de-differentiated, switched back on hTERT, which consequently resulted in re-expression of the α1,3Gal transgene.

These arguments could be individually addressed in an attempt to optimise FACS as a method for eliminating undifferentiated ES cells from a mixed population. For example, the use of homologous recombination, to target the α1,3Gal transgene into a site with characterised stable expression, would remove the risk of cells evading elimination through position effect variegation. By targeting the endogenous hTERT locus, this would increase the potential for *in vivo* regulation of tumours that arise after graft transplantation as the majority of tumours demonstrate hTERT expression (Tzukerman *et al,* 2000). Concurrent expression of α-gal in such modified ES cell derivatives, should invoke a rapid complement-mediated lysis response, following the emergence of only a small number of cells with tumorigenic potential, through naturally occurring anti-α-gal antibodies present in human serum. In addition, sort purity may be addressed by using stringent negative sort gates to prevent contamination of the negative population by partially differentiated cells with reduced expression. However, this would result in a significant reduction in yield of the negative, potentially therapeutic population, requiring significantly increased cell numbers to begin with, which would consequently increase cost and processing time, which could subsequently affect the viability of the therapeutic population.

Undifferentiated human ES cells are currently characterised using a panel of markers, including telomerase activity and TRA-1-81 expression. Therefore, it is hypothesised that future elimination experiments could benefit from combinational staining, with a number of "ES" specific markers, followed by enrichment for the population which is negative for all of them. During the course of this investigation, co-staining of cells partially differentiated for 6 days was performed with *BS-IB4* and Tra-1-81 (data not shown). Preliminary results from this population indicated that from the total population, 50.1% co-expressed both the α-gal and TRA-1-81 markers strongly suggesting that half of the population following 6 days in differentiation had the potential to have undifferentiated growth. Of the remaining 49.9%, 18.9% of the cells continued to express α-gal only and 5.3% continued to express TRA-1-81, while 25.7% of the population were negative for both markers. Using the current experimental design a single marker sort for TRA-1-81 could have contained as many as 18.9% of TRA-1-81 negative cells with continued undifferentiated potential, as indicated by the continued expression of hTERT driven α-gal. However, if the sorting strategy had been designed to collect cells that were double negative for both α-gal and TRA-1-81 it would have increased the stringency of the negative sort, increasing the purity of the potentially therapeutic population and thereby reducing the risk of tumorgenicity. The addition of a third selection marker, such as SSEA-3/-4 or transgenic Oct-4-GFP for example would further increase population purity, but as seen in the example above, cell retrieval would be significantly reduced with greater stringency, from 44.6% of the total population to 25.7%.

When differentiating human ES cells for clinical application, as well as considering the prevention of ES cell derived tumour growth, it is important to consider the number of cells that are required for therapeutic effect. For example, it has been suggested that in order to support liver failure in an adult, anywhere form 2-33% of the liver's cell mass would need to be replaced, equating, in humans, to between $1x10^9$ and $1x10^{11}$ cells per person (Haghighi *et al.,* 2004; Sundback & Vacanti 2000). Steps have been taken to improve the culture of human ES cells so that they are less labour intensive, in the absence of feeders and using enzymatic disaggregation, so that they would be more amenable to "bulk culture" (Thomson *et al.,* in preparation; Xu *et al.,* 2001; Amit *et al.,* 2000; Thomson *et al.,* 1998). In addition, a number of groups are investigating the possibility of expanding the currently small-scale lab based differentiation protocols to enable "bulk culture" for therapeutic use (Gerecht-Nir *et al.,* 2004 & 2004; Magyar *et al.,* 2001).

The implications of using multiple markers, whether they are specific for undifferentiated, or differentiated cell types, or a combination of both, to identify and eliminate a specific population would have significant implications for the volume of cells that would need to be produced to enable therapeutic applications, as already discussed. It is therefore unlikely that FACS could be extended into routine clinical practice as a single procedure for the elimination of potentially tumorigenic cells. A possible alternative to using multiple markers to increase the stringency of FACS would be to use a single marker but in multiple sorts. The first round of sorting would be used to significantly enrich the negative (or positive) population as presented here from ~10% to over 90%. This desired population would then be

returned to appropriate culture and expanded. Upon the second round of sorting there would be greater distinction between the two populations which would enable greater stringency of the sort gates which would increase the purity of the desired population without significantly effecting cell number. However, this technique would depend on the ability to expand differentiated cells *in vitro*, which is currently a limitation (Hay D. unpublished data).

Instead of using FACS as a substitute technique for the removal of undifferentiated human ES cells from a mixed population, it could be used in combination with complement-mediated lysis. In this chapter the potential of FACS as a strategy for evaluating markers of terminal differentiation and its ability to significantly enrich minority populations have been clearly demonstrated. Furthermore, α-gal under the control of hTERT has been shown to efficiently characterise the differentiation status of ES cells. However, despite the success of α-gal cell sorting, there appeared to be small populations of undifferentiated ES cells that were evading selective elimination. A similar situation was also reported when complement-mediated lysis was attempted on populations that had high-levels of ES cell contamination in Chapter 5. By combining the two techniques, the power of FACS would be utilised to enrich for therapeutic populations with great success, while the sensitivity of complement-mediated lysis to remove minority populations of contaminating undifferentiated ES cells would a) provide the purity and b) could be utilised *in vivo* as a form of immune surveillance.

7.4 Conclusion

For fluorescence activated cell sorting to be used as an alternative technique to complement-mediated lysis for the elimination of potentially tumorigenic undifferentiated human ES cells, either a true marker of the undifferentiated state of ES cells needs to be identified or else sorting should eliminate undifferentiated cells by sorting for the positive expression of specific markers of differentiated cells types. Preliminary data presented in this chapter indicate that expression of common markers used for identifying undifferentiated human ES cells, the tumour recognition/rejection antigens (TRA-), do not necessary indicate loss of undifferentiated growth potential. However, expression of transgenic epitopes under the transcriptional control of the human telomerase promoter (hTERT) indicated a much closer correlation to loss of undifferentiated growth potential.

FACS as a strategy for evaluating markers of terminal differentiation and for enriching minority populations has been clearly demonstrated but it is hypothesised that to achieve selective elimination of undifferentiated ES cells for clinical application, FACS may best be employed in combination with a second selective technique, such as complement-mediated lysis.

CHAPTER 8 DISCUSSION & CONCLUSIONS

The experiments described in this thesis were aimed at investigating strategies to eliminate undifferentiated, potentially tumorigenic, human ES cells from a mixed population of potentially therapeutic cells. Human ES cells were engineered to express a foreign epitope on their cell surface, which was down-regulated upon differentiation, thereby identifying those cells with undifferentiated growth/tumorigenic potential. This transgenic epitope was evaluated against cell surface markers endogenously expressed by undifferentiated human ES cells, reported to be down-regulated with differentiation, to compare their ability to identify and aid in the elimination of contaminating undifferentiated ES cells.

8.1 Summary of Results

Human ES cells were successfully engineered to express the α1,3galactosyltransferase (α1,3Gal) gene, responsible for expression of the major xenoantigen Gal α1-3Galβ1-4GlcNAc-R (α-gal) epitope, under the transcriptional control of the human telomerase reverse transcriptase (hTERT) promoter (Priddle,

H.). Although variegated, strong α-gal expression was found on cells in their undifferentiated state, at levels comparable to those observed on ovine (Polled-Dorset) foetal fibroblast (PDFF) cells with endogenous α1,3Gal expression. The M2 clonal cell line was identified as a strong candidate for further experiments due to its low level of variegation compared with other clonal lines, F11 for example. Transgenic α-gal epitopes on M2 cells were shown to bind natural anti-α-gal antibody, present in human serum, while wild type H9 cells remained non-reactive. Importantly, for the strategies reported in this thesis, transgenic α-gal epitopes were efficiently down-regulated upon differentiation and preliminary investigations indicating that loss of hTERT/α1,3Gal was more closely correlated to loss of undifferentiated growth potential than the characteristic ES cell marker, TRA-1-81.

In addition an attempt was made to engineer human ES cells to express the murine MHC molecule (H2-K^k) under the transcriptional control of the hTERT promoter. However, despite successful integration of the transgene (identified by RT-PCR), H2-K^k cell surface expression was not achieved and initial attempts to show protein translation, by Western Blot, were unsuccessful. It was suggested (Chapter 3) that issues associated with protein translation or with assembly and/or transport of H2-K^k from the endoplasmic reticulum were responsible for the lack of cell surface expression. However, the potential requirement for additional genes, such as β-2 microglobulin (β-2M) or possible stimulation with interferon-gamma (IFN-γ) to overcome this issue suggested that H2-K^k was not as suitable as α-gal as a lysis epitope for therapeutic application.

Results presented in Chapter 3 indicated that the use of trypsin/EGTA (TEG) as an enzymatic method for disaggregating human ES cells in routine culture generated a more homogeneous population of human ES cells, as compared with collagenase IV disaggregation, with little, if any differentiation. Human ES cells cultured in this way, dissociate into a single cell suspension, aiding both of the elimination strategies and the optimisation of electroporation techniques. Furthermore, the ability of engineered cells to proliferate or differentiate was unaffected and the cells maintained a normal karyotype through over 40 passages. It is possible that previous karyotypic abnormalities observed in the ES cells maintained by the McWhir group whilst using TEG dissociation (Thomson *et al.,* in preparation), resulted from a unique selection pressure that was exerted as part of their *in vitro* culture, unrelated to the use of TEG. This suggests that perhaps the conditions in which human ES cells are currently maintained require further optimisation.

Strategies to eliminate undifferentiated ES cells focused on the use of complement-mediated lysis in Chapters 4 and 5. Using PDFF cells as a positive control the Calcein-release assay was optimised for the detection of α-gal induced lysis of M2 and F11 ES cells in normal human serum. These optimisation assays clearly indicated that different cell types respond differently to the Calcein label and that the use of sub-optimal procedures could result in inappropriate interpretation of results. Following optimisation, the Calcein-release assay (using 40μM Calcein labelling) was shown to be an extremely reliable method of detecting complement-mediated lysis. In turn complement-mediated lysis (using undiluted human serum) was shown to be a very efficient method for repeatedly eliminating minority populations of

undifferentiated ES cells which expressed the α1,3Gal transgene. In the case of M2-ES cells, α-gal induced complement-mediated lysis occurred at the same level as observed for endogenously expressing PDFF cells. Interestingly when the level of M2-ES cell contamination was 0.8% of the total population, no residual ES cell colonies were observed. However, with increased levels of contamination, the number of undifferentiated M2-ES cells that avoided elimination increased steadily, showing frequencies of <0.07% residual cells at 3% contamination, increasing to <1.33% at 25% contamination, <2.60% at 50% contamination and <3.80% from a pure population of undifferentiated M2 ES cells treated with human serum. Although variegated transgene expression could have accounted for some of these residual cells, it is considered that saturation of the experimental conditions would explain the gradual increase in the number of surviving cells as the level of contamination increased. It is hypothesised that if serum treatment had been repeated, these residual cells could have been eliminated. Importantly, differentiation of α-gal expressing ES cells (M2) resulted in a significant reduction in their sensitivity to complement-mediated lysis. After 21-days in differentiation medium only 8% of the cells remained sensitive to complement, which correlated to the proportion of cells that continued to express α-gal, as determined by flow cytometry (Chapter 7) at a similar time point.

During the optimisation of the complement-mediated lysis assay, it was hypothesised that due to the embryonic nature of ES cells and evidence that cells of the human blastocyst express high levels of membrane bound complement regulators (Fenichel *et al.,* 1995), it was possible human ES cells could be protected from complement-

mediated lysis by the presence of complement regulatory proteins. Evidence was provided (Chapter 4) to show that undifferentiated ES cells (H1, H7 & H9) express high levels of the complement regulatory proteins to similar (CD59) or greater (CD55) levels than on human multipotential erythroid progenitor cells (K562). Furthermore, complement regulators were expressed at similar levels on Human Embryonic Kidney (HEK) 293 cells, suggesting that this level of expression was due to the embryonic nature of the cells and not because they were ES cells *per se*. However, high expression levels of CD55 and CD59 were insufficient to inhibit complement-mediated lysis of human ES cells, although it remains unknown if increased levels of CD55 and CD59 could protect undifferentiated ES cells from low level attacks as discussed in Chapter 4. In addition to serum, containing antibody, the use of exogenous antibody to an endogenous ES cell surface epitope was also shown to be capable of eliciting a complement-mediated attack, providing the opportunity to develop this technique in conjunction with vaccination strategies.

In Chapters 6 and 7 the focus of elimination switched to using fluorescence activated cell sorting (FACS) as an alternative technique to complement-mediated lysis to remove undifferentiated ES cells from within a mixed population. Enrichment of minority populations by FACS was clearly demonstrated and importantly the process of sorting was shown not to affect the viability, pluripotentiality or karyotypic stability of undifferentiated ES cells. These data confirmed the assumption made in the report by Eiges *et al.*, 2001. In addition preliminary results (Chapter 6) demonstrated that FACS could be used to single cell sort undifferentiated human ES cells and that these cells survive and proliferate at a frequency of 10.4%. However,

although the majority of these cells maintained an undifferentiated ES cell phenotype, further experiments are required to determine if these cells maintain the characteristic properties of undifferentiated ES cells.

As reported in Chapter 7, under the conditions used in this thesis, FACS, using α-gal and TRA-1-81 as markers, was unable to separate a population of cells that was free of undifferentiated ES cell growth potential. Evidence was provided to show that FACS could be used to greatly increase the purity of an indiscriminately stained population. However, specifically in the case of TRA-1-81, a comparison of the negative (91.5% cells) and positive (94.2% cells) populations indicated that a similar level of ES-like cell growth occurred in both populations. These data strongly suggest that, contrary to current perception, TRA-1-81 is a poor marker of ES cell differentiation, thus highlighting the importance of verifying dogma reported in the literature (Draper *et al.,* 2002). By contrast, the negative α-gal population contained far fewer undifferentiated ES-like cells compared to positive α-gal population, suggesting that hTERT/α-gal expression was significantly better at discriminating between the undifferentiated and differentiate status of ES cells. It is hypothesised that small adjustments to the sort parameters in subsequent experiments would be sufficient to eliminate these cells, however, restricting sort gates has the potential to increase the cell number requirement and the level of cell wastage, thus increasing cost and processing time which could affect viability. It is considered that for FACS to be used to eliminate potentially tumorigenic undifferentiated human ES cells as a stand alone, single use strategy either a true marker of undifferentiated ES cells

needs to be identified or else sorting should eliminate undifferentiated cells by sorting for the positive expression of specific markers of differentiated cells types.

8.2 Critique of this Research

The majority of the work reported in this thesis reflects the use of a clonal human ES cell line, M2. Although other clonal cell lines were investigated, all lines were derived from the H9 parental ES cell line. To support the potential of this strategy for clinical development, other parental human ES cell lines, such as H1 and H7, would need to be engineered to determine if the regulation of hTERT/α1,3Gal expression is comparable and that the effects of complement-mediated lysis against the α-gal epitope are reproducible in multiple cell lines. In addition M2 and F11 clonal cell lines expressed hTERT/α1,3Gal from random integration sites, which as reported in Chapter 3 clearly experienced varying degrees of variegation. It has been hypothesised that position effect variegation (PEV) may have been responsible for some undifferentiated ES cells evading elimination under the strategies reported here. As previously discussed (Chapters 3 & 7) targeting the α1,3Gal construct into the endogenous hTERT locus, for example, to acquire the native hTERT promoter should eliminate the effects of silencing elements and heterochromatinisation. However, there is a risk that disrupting a copy of the hTERT gene could affect the ability to maintain ES cells indefinitely in an undifferentiated state and could potentially increase cellular aging of the differentiated derivatives if hTERT functions in a dose-dependent manner. Alternatively, the hTERT/α1,3Gal expression vector could be targeted into a "neutral" genomic site, un-associated with PEV, or else the incorporation of insulator elements, to confer site-independent expression,

could be investigated (Burgess-Beusse *et al.,* 2002; Hasegawa & Nakatsuji 2002). In addition there is the potential to use lenti-viral vectors, which have been reported to be free of the effects from gene silencing (Martin *et al.,* 2005; Gropp *et al.,* 2003; Ma *et al.,* 2003), as a method of engineering the ES cells.

During the course of this work non-specific differentiation protocols have been adopted to determine the global regulation of α-gal with differentiation. It is possible that continued α-gal expression at the latter time points was as a consequence of persistent hTERT expression. Although telomerase is considered a characteristic of embryonic, germ-line (Wright *et al.,* 1996) or malignant tissues (Tzukerman *et al.,* 2000), there is a growing body of evidence that shows expression of telomerase in some somatic cell types including, haematopoietic progenitors, particularly lymphoid lineage committed progenitors, normal peripheral blood leukocytes (Hohaus, *et al.,* 1997; Weng, *et al.,* 1996) and in the continuously proliferating epidermis basal layer (Harle-Bachor *et al.,* 1996). In terms of regenerative medicine it is likely that partially differentiated, progenitor cells will be the targets for engraftment to enable the graft to proliferate *in situ*, to aid integration and regeneration in the host. It will therefore be important to investigate the persistence of hTERT expression using specific differentiation protocols, such as osteogenic or neural lineages for example, to determine if hTERT expression is present in progenitors. It will also be important to assess the use of alternative ES-restricted promoters, such as Oct-4 or Rex-1, (reviewed by Ginis *et al.,* 2004) to restrict expression of α-gal to only those cells with undifferentiated growth/tumorigenic potential, should hTERT prove not to be efficiently restrictive.

For any elimination strategy to be of therapeutic relevance, it is essential that the strategy can be widely implemented, providing significant advantage to a large number of individuals. As discussed in Chapter 1, there is evidence to suggest that specificity of the anti-α-gal antibody is blood group dependent (Galili *et al.,* 1987b). It has been reported that approximately 75% of the anti-blood group B antibody found in sera of blood group-A and -O individuals cross reacts with the α-gal epitope due to similarities in their structure (Galili *et al.,* 1987). The experiments reported in this thesis show the level of complement-mediated lysis as a result of exposure to pooled serum from blood group-A individuals. Although there is no evidence that serum from blood group-B individuals has lower levels of specific anti-α-gal antibody to those found in blood group-A & -O serum, serum from blood group-B and -AB individuals lack the additional cross reactive anti-B antibody (Oostingh *et al.,* 2003; McMarrow *et al.,* 1997; Galili *et al.,* 1984). It is therefore likely that although complement-mediated lysis would still occur following exposure to blood group-B serum the efficiency would be reduced, possibly requiring increased exposure time or multiple rounds of treatment. The blood group-B antigen is the least common of the blood group antigens, with blood types AB and B accounting for approximately 15% of all the blood types. However, in the areas of Eastern Europe and Central Asia the B-antigen is more common, and can account for up to 38% of the populations (www.bloodbook.com/world-abo.html, 2005). Therefore, an assessment of different blood types must be made before such a strategy could be considered for therapeutic use. If exposure to blood group-B or -AB serum proves unable to eliminate undifferentiated ES cells efficently then the situation could, in

principle, be redressed by immunisation against α-gal for *in vivo* strategies. *In vitro* strategies are not sensitive to blood type, but it would be beneficial to use serum from blood group-O individuals, since blood group-O serum has the broadest range of anti-α-gal antibody activity due to the present of cross reactive anti-blood group-B and -A antibodies (Galili *et al.,* 1987b).

8.3 Potential Clinical Application of hTERT/α1,3Gal Expressing Human ES Cells.

The tumorigenic potential of undifferentiated ES cells is significant following subcutaneous injection into SCID mice as discussed in section 1.4 (Reubinoff *et al,* 2000; Thomson *et al,* 1998; Thomson *et al.,* 1995; Evans & Kaufman, 1981). However, the level of ES cell contamination of a therapeutic population that would result in tumour formation remains unknown. It has been reported that tumorigenesis can be induced by as few as 500 undifferentiated, Oct-4 positive murine ES cells, irrespective of the site of implantation (Erdo *et al.,* 2003). Furthermore, pre-differentiation of such cells into neural progenitors, containing less than 0.5% Oct-4 positive cells, resulted in 86% of the mice developing tumours within 2-weeks of transplantation (Erdo *et al.,* 2003).

Chapters 4 to 7 have assessed the individual merits of both complement-mediated lysis and of fluorescence activated cell sorting (FACS) as alternative methods for the removal of contaminating potentially tumorigenic undifferentiated human ES cells, based on the specific expression of transgenic α-gal or endogenous TRA-1-81 epitopes. As indicated in Chapter 7, α-gal epitopes under the control of the hTERT

promoter were efficiently down-regulated upon differentiation and displayed a closer correlation to loss of undifferentiated growth potential than the endogenous human ES cell marker TRA-1-81.

In addition, the use of hTERT/α1,3Gal to provide unique α-gal epitopes on the cell surface of undifferentiated ES cells has the advantage that humans have natural immunity to α-gal; human serum contains high resting levels of anti-α-gal antibody (30-70μg/ml; Galili *et al.,* 1984, 1985). Naturally high levels of circulating antibody have the potential to provide an *in vivo* surveillance system, not only against the accidental transplantation of undifferentiated ES cells, but also against cells which dedifferentiate, or which become malignant *in vivo*, with reactivation of the hTERT promoter occurring in over 80% of studied malignancies (Tzukerman *et al.,* 2000). The development of malignancy would result in re-expression of α1,3Gal by these cells and re-presentation of the α-gal epitope on the cell surface.

The prevention of tumour growth following the use of ES-derived cell therapy is an important safety consideration; however, in eliminating this risk, it is important that strategies take into account the number of cells that will be required for therapeutic application and how *in vitro* research can be adapted for clinical use. As discussed in Chapter 7, cell numbers in the region of $1x10^9$-$1x10^{11}$ per person have been reported for the treatment of adult human liver failure (Haghighi *et al.,* 2004; Sundback & Vacanti 2000). This thesis presents "proof of principle" data showing complement-mediated lysis on a small scale to eliminate contaminating ES cells, requiring 100μl of human serum to treat 10^5 cells. Translated into clinical terms this would be

equivalent to 1-100 litres of serum (approximately 5-500 litres of human blood) to eliminate undifferentiated ES cells from the 10^9-10^{11} liver cells required to treat one liver patient. Since there is already a shortage of blood the requirement for these quantities of human serum is unsustainable. Furthermore, the challenge of maintaining and regulating the quality of serum to ensure good manufacturing practice (GMP) over time for clinical application, including the necessary health and safety issues, pathogen screening etc, suggests that this strategy would not be viable on such a large scale. To overcome these issues it is possible that a human monoclonal antibody could be developed, which when used together with recombinant/purified human complement would substitute the requirement for human serum/blood. However, this technique would require significant optimisation, is likely to be very expensive and again could prevent large-scale rollout on the National Health Services (NHS).

FACS, as an alternative, has the ability to handle large volumes of cells. However, while FACS showed significant enrichment of α-gal negative populations from 10.3% to 97.3%, not all cells with undifferentiated ES growth potential were removed. To increase the purity of therapeutic populations sorted by FACS, multiple markers, either specific for undifferentiated or differentiated cell types or combinations of both could be used, or else tighter sorting parameters or multiple rounds of sorting could be tried. Whilst such strategies would improve the purity of the resulting population, they could significantly reduce the yield of the potentially therapeutic population, which would have a significant impact on the number of cells

required to begin with, consequently increasing cost and processing time, the latter subsequently affecting the viability of the therapeutic population.

It is therefore unlikely that, as with complement-mediated lysis, FACS could be extended into routine clinical practice as a single procedure for the elimination of potentially tumorigenic cells. A strategy that would be worth considering is the use of magnetic activated cell sorting (MACS) in combination with either FACS or complement-mediated lysis, or both. *Miltenyi Biotec* (GmbH, Germany) has developed *CliniMACS*®, which is a system that can be used to deplete or enrich for specific sets of cells on a large scale, through magnetic labelling of cell surface epitopes. This unique system has been used clinically to enrich for $CD34^+$ cells from peripheral blood, without affecting the viability of the cells (Despres *et al.,* 2000). The advantage of *CliniMACS*® over FACS is the size and cost of the equipment, and the quantity of cells that *CliniMACS*® can process; it has the capacity to sort some $4x10^{10}$ cells in approximately 2 hours, within a sterile environment. It is proposed that, by combining the enrichment power of a technique such as FACS or MACS with the sensitivity of complement-mediated lysis to effectively eliminate low-level contamination, this strategy would prove to be very powerful at eliminating undifferentiated ES cell contaminants, specifically with the potential for *in vivo* surveillance as a result of α-gal.

As discussed in Section 1.6.2 natural immunity to α-gal expression holds great promise for the treatment of cancer. A number of reports have shown that different human cancer cell lines can be efficiently eliminated by human serum following

transfection/transduction with the α1,3Gal gene and their ability to establish tumours following treatment is reduced (Aubert *et al.,* 2003; Unfer *et al.,* 2003; Sawada *et al.,* 2002; Yoshimura *et al.,* 2001; Jäger *et al.,* 1999; Link *et al.,* 1998). In addition, LaTemple *et al.,* (1999), immunized α1,3Gal knockout mice with irradiated tumours cells that were engineered to express α-gal on their cell surface and were able to show that the α-gal epitope acted as an opsonin, encouraging phagocytosis. The use of α-gal as a mechanism to initiate cell death directly in transfected cells or to indirectly target cells through immunization, provides evidence that the response to the presence of α-gal epitopes is not restricted to the endothelium of vascularised tissues and organs, underlying the potential for *in vivo* immune surveillance. However, in an allograft setting, it will be important to establish that the level of immune suppression necessary to prevent graft rejection would not entirely eliminate circulating anti-α-gal activity.

8.4 Taking this Research Forward - Future Experiments

In addition to the experiments described above (see section 8.2) to improve the reproducibility and potential of the techniques described in this thesis, there are a number of interesting experiments that can be performed with the existing cell line (M2). As discussed it will be important to use specific, directed differentiation protocols to assess expression levels of hTERT/α1,3Gal in potentially therapeutic progenitor cells. In doing so this will determine a time point at which cells with undifferentiated growth potential can be eliminated without eliminating those cells of potential therapeutic value.

In collaboration with labs that have optimised specific differentiation protocols for human ES cells, and for whom the occurrence of tumours in transplantation studies are proving to be problematic, it would be interesting to test the *in vitro* elimination strategies described in this thesis. The aim of these experiments would be to ascertain whether treatment of potentially therapeutic cells with complement or sorting populations by FACS, affects their ability to engraft, integrate, migrate and provide functional improvement to the same level as untreated controls. Differentiated derivatives of M2-ES cells which have undergone either treatment with human serum or cell sorting or a combination of both, to remove potentially tumorigenic cells (referred to as stripped populations) would be injected into SCID mouse models. To reflect the same environmental stresses that the stripped populations had gone through, controls of un-stripped populations, which had been sorted, based only on viability, or treated with heat-inactivated human serum or a combination of both, would be used. The development of tumours would then be used, in combination with a panel of screening assays (such as analysis or expression markers and undifferentiated ES cell growth *in vitro*) as a measure of the success of hTERT/α1,3Gal, and the respective elimination strategies to prevent tumour formation compared to the unstripped controls and an experimental standard, untreated control.

In the long-term it would be interesting to investigate the potential of *in vivo* α-gal immunity against residual ES cells which express hTERT/α1,3Gal. To achieve this a mouse model which has the α1,3Gal knockout phenotype (Ohshima *et al.,* 1997; Thall *et al.,* 1995) and also the ability to accept human cell transplants needs to be

created. Mice that possess the SCID mutation have no acquired immunity, lacking both B- and T- lymphocytes they are unable to produce antibody or initiate humoral immunity (reviewed by Bancroft & Kelly, 1994). When the SCID mutation is in the C57BL/6 or Balb/c genetic background, the mice retain a functional innate immune system with normal levels of macrophages, natural killer (NK) cells and antigen presenting cells (APC), in addition to normal, sometimes elevated complement activity (JAX® Communication 2000; reviewed by Bancroft & Kelly, 1994). On the other hand, NOD inbred mice have impaired, macrophage, APC and NK cell function and lack haemolytic complement C5, and so NOD-SCID mice are complement deficient (JAX® Communication 2000). Therefore, to create a model in which to test *in vivo* immunity against the M2 human ES cell line SCID mice of appropriate genetic background (C57BL/6 or Balb/c) should be crossed to the α1,3Gal knockout mouse (Ohshima *et al.,* 1997; Thall *et al.,* 1995) and subsequent offspring inbred to produce a stable SCID/α1,3Gal knockout mouse.

Transplantation of M2 human ES cells into this model, with sufficient quantities of anti-α-gal antibodies (requiring repetitive immunisation over time) could be used as a model to test the effectiveness of *in vivo* complement-mediated lysis against residual undifferentiated ES cells following transplantation. Initially it would be interesting to use the SCID/α1,3Gal knockout to perform dilutions studies, to determine the level of ES cell contamination that the *in vivo* immune system would be able to clear, without tumour formation. To achieve this undifferentiated M2 (α-gal expressing) cells would be mixed with differentiated, (non proliferative, non-α-gal expressing) cells at serial dilutions. Pure ES and pure differentiated cell populations would be

used as positive and negative controls respectively and the occurrence of tumours and *in vitro* ES cell culture etc would be used as a measure of success. The aim of these experiments would be to determine the minimum level of purity that would be required from *in vitro* elimination strategies and would provided an insight into levels of risk.

8.5 Conclusion

The novel system for selective ablation reported in this thesis could potentially provide natural immune protection to eliminate the risk of tumorgenicity associated with ES-derived therapy. Naturally circulating antibodies to α-gal could protect graft-recipients from the presence of, or de-differentiation of, human ES-cell derivatives following engraftment. Combining the sensitivity of complement-mediated lysis at eliminating minority populations with the enrichment power of fluorescence activated cell sorting (FACS), or magnetic activated cell sorting (MACS), would potentially overcoming issues of scalability, that could otherwise hamper this technique in its move from research bench to widespread clinical application.

The advantage of the hTERT/α1,3Gal strategy over the use of constitutive HSV-tk expression, report by Schuldiner *et al.* (2003), is that by restricting expression of α1,3Gal through the use of the hTERT promoter, only areas of the graft that become tumorigenic will be eliminated *in vivo*. Thus preventing representation of symptoms and the need for further intervention. However, this strategy does not address the issue of post-transplant complications, unrelated to tumorigenicity, which might affect the whole ES-cell derived graft. Therefore, it is likely that these two strategies, hTERT/α-1,3Gal for tumour elimination, and constitutive HSV-tk, in case of post-graft complications, would benefit from being combined.

Whilst investigating the potential of complement-mediated lysis and FACS to eliminate undifferentiated ES cells, this thesis has brought to light a number of interesting observations.

1. It provides evidence to show that clonal ES cell lines can be cultured using enzymatic disaggregation techniques (TEG) for many (>50) passages without effect on their karyotypic stability;
2. Evidence has been provided that indicates that undifferentiated ES cells express high levels of complement inhibitory proteins, in line with expression on the human blastocyst (Fenichel *et al.,* 1995);
3. Undifferentiated ES cells have been shown to survive and proliferate following single cell sorted; and
4. It has challenged the use of TRA-1-81 as a characteristic marker of undifferentiated ES cells, by showing that its down regulation is not indicative of loss of undifferentiated growth potential as reported in the literature (Draper *et al.,* 2002).

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APPENDIX I CONSTRUCTS & CLONING STRATEGIES

I.1 H2-K^k - A Murine Major Histocompatability Complex Antigen

Initially there were two transfection strategies, one based on the use of the Oct-4 promoter and the other based on the hTERT promoter. Both of these promoters have the unique property of being down regulated in human ES cells, upon initiation of differentiation. In both strategies the H2-K^k gene was cloned by high-fidelity PCR using *PfuTurbo* DNA Polymerase (Stratagene) using the commercially available plasmid pMAC KK II (Milteny Biotech) plasmid DNA as a template, (see Table I.1.1 for details).

Primers	50µl Reaction Mix		PCR Machine/Program
Fwd Primer: GATGGCACCCTGCATGCTGCT **Rev Primer**: GGATCTACCCTCCTTTTCCACCTGTGTTTC	Template DNA	10ng	*Hybaid MBS 0.2*
	PCR Buffer	10X	
	F. Primer	100pM	60s at 95°C
	R.Primer	100pM	30s at 95°C } 20
	$MgCl_2$	1mM	60s at 63°C } 20
	Mixed dNTPs	200µM	60s at 72°C } 20
	PfuTurbo	25Units	180s at 72°C
	Sterile dH_2O	to 50µl	

Table I.1.1: High fidelity PCR Conditions for Cloning the H2-Kk cDNA from the pMAC Kk II Plasmid (Milteny Biotech). Primers were produced by MWG.

The blunt ended H2-K^k PCR product produced using *PfuTurbo* was then treated with Taq DNA polymerase (Roche), to add a single deoxyadenosine to the 3'-ends of the amplified fragment to enable efficient cloning into the TA vector system (pGEM-Teasy Promega). The advantage of the pGEM-TEasy vector system was the multiple cloning sites at each end of the integration site, which provided a selection of restriction enzymes for future cloning strategies.

The expression vector (phTpAPN) was built (Priddle, H. Roslin Institute) using a core (200bp) human telomerase reverse transcriptase (hTERT) promoter and upstream transcription termination signal (kind gift of Geron Corp, USA) followed by a multiple cloning site for cDNAs and a bovine growth hormone poly-adenylation signal (kind gift of Geron Corp, USA). The vector also contained a constitutive neomycin resistance gene and poly-adenylation signal driven by a PGK promoter to provide selection in eukaryotic cells. The H2-K^k PCR product was excised from the pGEM-TEasy Vector with *EcoRI* and ligated into the *EcoRI* site within the multiple cloning site of phTpAPN to produce phTH2KkpAPN (Figure I.1.1A). The constitutive neomycin cassette was used for selection of stable transfectants.

Expression of H2-K^k under the transcriptional control of the human Oct-4 promoter was achieved in a similar manner. The phOCT-4-eGFP-1 plasmid (a kind gift from Debiao Zhao, Roslin Institute) was digested with *AgeI* (sticky) and *HpaI* (Blunt) to remove the eGFP-1 mini gene. The *AgeI* 3' receded end was filled using Klenow (*NEB*) and the H2-K^k PCR product cloned into the phOCT-4 vector backbone as an *EcoRI* blunt fragment, which was excised from pGEM-TEasy with *EcoRI* and

blunted with Klenow. The resulting construct, phOct-4-H2-K^k contained a constitutive neomycin selection cassette for selection in eukaryotic cells (Figure I.1.1B).

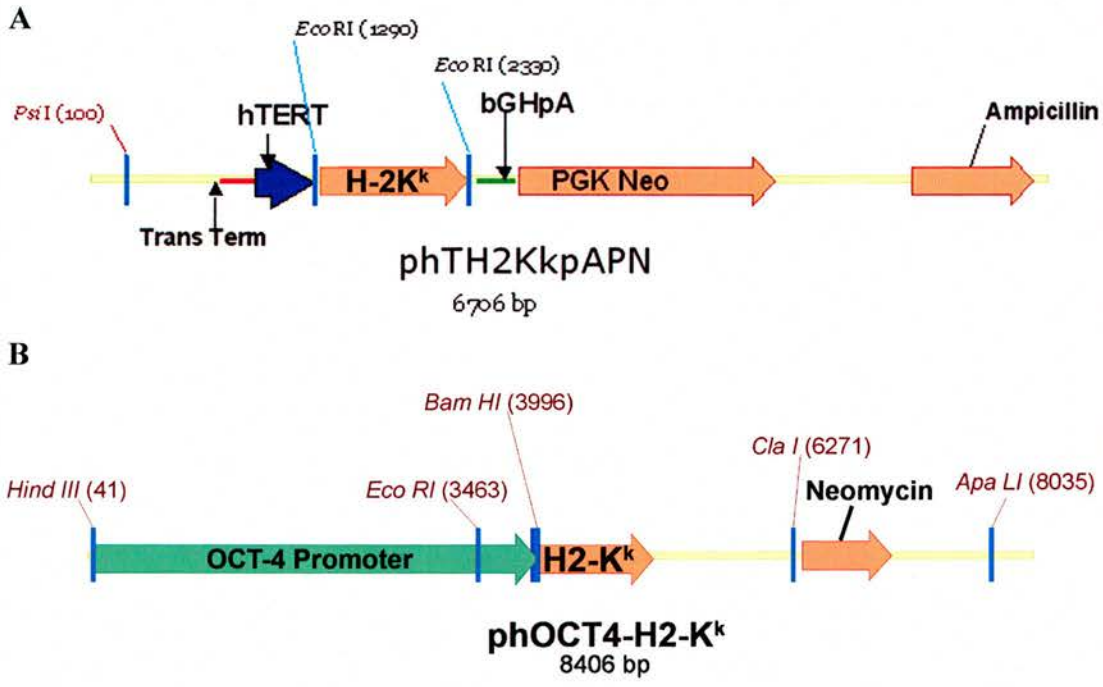

Figure I.1.1: A schematic representation of the H2-Kk expression constructs. A] H2-Kk was cloned by PCR from pMAC KK II (Milteny Biotech) and TA cloned into pGEM TEasy (Promega) before being subcloned as an Eco R1 fragment into phTpA PN (Priddle H). PsiI was used as a site for linearising the construct. B] using an AgeI : HpaI digest the eGFP minigene was removed from phOCT-4-eGFP (Zhao D) and the H2-Kk PCR product subcloned in as a blunted EcoR1 fragment. Apa LI was used as a linearisation site.

In the first instance phTH2-KkpAPN was linearised (psiI) and stably transfected into 10^6 wild type H7 human ES cells by electroporation (multiporator, Eppendorff) as described in section 2.7.2. Cells were plated under 200µg/ml G418 selection for 7 to

10 days, after which the resistant colonies were pooled and the resulting cell line was referred to as H7-K^k.

I.2 Galα1-3Galβ1-4GlcNAc-R (α-gal) Epitope

The α1,3galactosyltransferase (α1,3Gal) expression vectors used in this study were constructed by Dr. Helen Priddle (Roslin Institute, Edinburgh). Briefly, mouse kidney mRNA was used to amplify the α1,3Gal coding sequence by reverse transcription (RT) PCR using 5' and 3' primers outlined in Table I.2.1. The resulting coding sequence was designated mGT. A fucosyltransferase (FucT) leader sequence was engineered at the N terminus of mGT prior to the transmembrane domain, following Osman *et al.* (1996), by engineering PCR primers which contained the desired leader sequence of the fucosyltransferase gene before performing PCR on the mGT cDNA (primers described in Table I.2.1). Following PCR, both mGT and FucGT were TA cloned into pGEM-TEasy (Promega)

Coding sequence	5' Forward Primer	3'Reverse Primer
MGT	ggcctgtactacatttgcctgga	gaaatagtgtcaagtttccatcacaa
FucGT	cgatgtggctgcggagccaccggcaggtaatcctgttgatgctgattgtctcaac	gaaatagtgtcaagtttccatcacaa

Table I.2.1: Primer design for cloning of mouse galactosyltransferase and the leader sequence of the fucosyltransferase gene by RT-PCR.

Both the mGT and fucGT coding sequences were excised from pGEM-TEasy with *EcoRI* and were subcloned into the *EcoRI* site contained within the multiple cloning site of phTpAPN. As described above, (see section I.1) phTpAPN contained the

hTERT core promoter (kind gift from Geron corp.) to provided restricted expression of α1,3Gal to undifferentiated ES cells (Figure I.2.1).

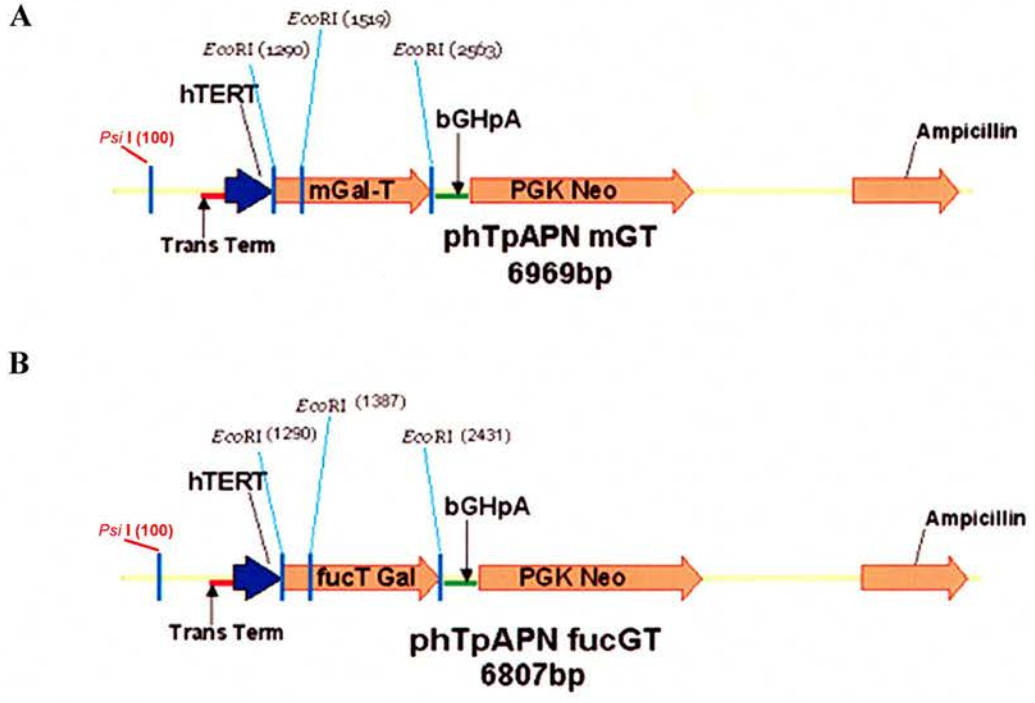

Figure I.2.1: Schematic representation of the two constructs used to express the galα1-3Galβ1-4GlcNAc-R (α-gal) epitope of the cell surface of human ES cells. Both constructs were engineered by Dr Helen Priddle, a] mouse kidney α1,3galactosyltransferase cDNA under the control of a core hTERT promoter b] the same construct containing the leader sequence of the fucosyltransferase gene.

Dr H. Priddle produced transgenic mGT and fucGT cell lines, by electroporation using hyposmolarity buffer (Eppendorf) and an Eppendorff Multiporator (Eppendorf). Briefly, $1x10^6$ H9 human ES cells were electroporated with linear DNA, plated and selected in the presence of 200μg/ml G418 for 10-14 days. From each transfection, yielding 558 and 585 colonies respectively, 24 colonies were

picked and expanded, from which 4 of the mGT clones and 8 of the fucGT clones expressed the α-gal epitope.

I.3 The Undifferentiated ES Cell Reporter Construct - mOct-4-EGFP

The reporter construct mOct-4-EGFP was a kind gift from Dr Ed Gallagher (Roslin Institute, Edinburgh) and remained unchanged (Figure I.3.1).

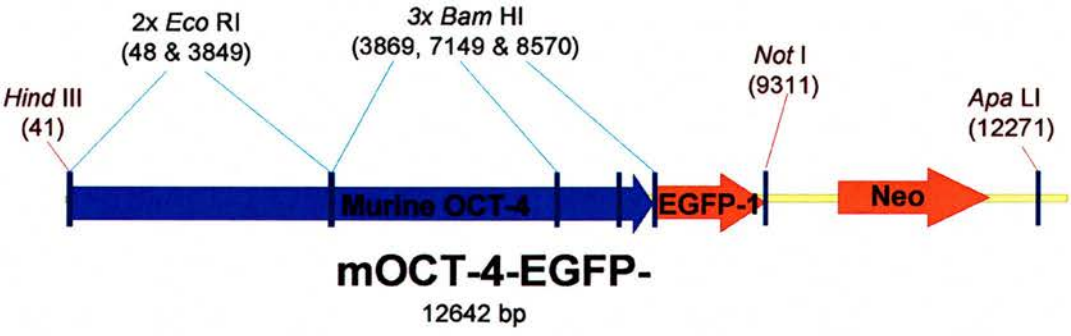

Figure I.3.1: A schematic representation of the Oct-4/GFP expression vector, a kind gift from Dr Ed Gallagher. The reporter construct contains the full 8.5kb murine Oct-4 promoter driving the wild type EGFP reporter gene. Stable cells lines were created by lipofection, using Apa LI linearised DNA, and were selected for with 200μg G418.

The mOct-4-EGFP construct was linearised (*ApaL1*) and transfected into H9 human ES cells by Lipofection (Lipofectamine 2000, Invitrogen) as described in section 2.7.1. G418 Selection (200μg) was applied 48-hours after the transfection agents were removed, and was maintained for 7 to 10 days, after which, 9 colonies were picked and transferred into a 24-well plate. Of the 9 picked colonies, 6 were successfully expanded into stable cell lines, but they displayed varying degrees of

transgene expression. Oct-4/GFP-6 showed the lowest level of variegation and was therefore the clone of choice for subsequent experiments.